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Role of Iron in the Regulation of Phenylalanyl-tRNA Synthetase Activity in *Azotobacter vinelandii*

by

Manisha Mehrotra ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ROLE OF IRON IN THE REGULATION OF PHENYLALANYL-tRNA SYNTHETASE ACTIVITY IN *AZOTOBACTER VINELANDII* submitted by MANISHA MEHROTRA in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY. in MICROBIOLOGY & BIOTECHNOLOGY

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To

Mummy and Papa

For their love, support and encouragement over the years

ABSTRACT

Azotobacter vinelandii strain UA22, a Tn*5luxAB* insertional mutant of wild type strain UW, is characterized by its iron-regulated bioluminescence. The site of the Tn*5luxAB* insertion was localized by subcloning and sequencing. The sequence of this region (1.541 kb of DNA) revealed an ORF that is 72% identical to the 3' end of the *Escherichia coli rplT* gene (encoding the ribosomal protein L20), followed by an ORF that is 63% identical to the *E. coli pheS* gene (encoding the alfa subunit of the phenylalanine-tRNA synthetase enzyme), which is interrupted at the 3' end by *luxA* (coding for the alfa subunit of luciferase). Promoter and attenuator sequences, similar to those located upstream of the *E. coli pheS* gene, were not found in the *A. vinelandii rplT-pheS* intergenic region. Two putative (overlapping) iron-boxes, which shared 53%-58% identity with the *E. coli* consensus sequence, were located about 390 bp into the *A. vinelandii pheS* gene. A fragment of *pheS* containing these iron-boxes was isolated, amplified using PCR, and shown to have iron-regulated promoter activity in the promoter probe vector pQF50. Primer extension analysis identified a possible transcription start point within the *pheS* gene. Sequences showing weak homology to the *E. coli* σ^{70}-like -10 and -35 promoter determinants were identified.

Isolation of *pheS*-disruption mutant was possible because *A. vinelandii* is polyploid and the mutant *pheS*::Tn*5luxAB* allele exists in very few chromosomes of this organism. The UA22 DNA fragment was shown to bind purified Fur (for ferric uptake regulation) from *E. coli* in gel retardation assays. A Fur-like protein that cross-reacted with anti-*E. coli* Fur and anti-*Pseudomonas* Fur antiserum was identified in *A. vinelandii* by Western blotting. The tRNA charging activity of phenylalanine-tRNA synthetase of *A. vinelandii* UW cell extracts, using ^{14}C-labeled phenylalanine, was found to be up-regulated 1.4 to 1.6-fold by iron depletion.

A model is proposed in which a Fur-binding site within the the *pheS* gene *of A. vinelandii* can either function as a road-block in the transcriptional elongation of *pheST* mRNA, initiated from a promoter in front of *rplT*, or can block the initiation of new transcript, starting within the *pheS* gene from the weak promoter identified in this study.

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List of Abbreviations

+Fe	high-iron
-Fe	low-iron
A_{310}	absorbance at 310 nm
A_{380}	absorbance at 380 nm
A_{420}	absorbance at 420 nm
A_{550}	absorbance at 550 nm
A_{600}	absorbance at 600 nm
BBGN	Burk's buffer with glucose and nitrogen source
BCIP	5-bromo-4-chloro-3-indolyl phosphate-p-toluidinium salt
Bistris	bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
bp	base pairs
ddATP	dideoxy adenosinetriphosphate
ddCTP	dideoxy cytosinetriphosphate
ddGTP	dideoxy guanosinetriphosphate
ddTTP	dideoxy thyminetriphosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
EDDHA	ethylenediamine-di-(o-hydroxyphenyl)acetic acid
EDTA	ethylene diamine tetraacetic acid
FAB	fast atomic bombardment
h	hour(s)
IPTG	isopropyl β-D-thiogalactopyranoside
Kan^R	kanamycin resistant
µCi	microCurie
mCi	milliCurie
µF	microFaraday
min	minute(s)
µM	micromolar
mM	millimolar
NBT	nitro-blue-tetrazolium

nM	nanomolar
OFe BBGN	BBGN without iron
ONPG	o-nitrophenyl-β-D-glycopyranoside
ORF	open reading frame
PEG	polyethyleneglycol
rbs	ribosome binding site
RNase	ribonuclease
SDS	sodium dodecyl sulfate
sec	seconds
SSC	sodium chloride (3M) in 0.3 M tri-sodium citrate
TCA	trichloro acetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer RNA
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1
General Introduction

1.1 The importance of iron in all life forms

Iron is a prominent element on earth and in the entire solar system. Among all elements on the surface of planet earth, it ranks fourth in abundance and, among metals, is second only to aluminum (Lankford, 1973). Iron has the ability to be oxidized and reduced at physiological pH, which makes it a prime electron transporter. Iron plays a key role in a variety of vital functions performed by a cell. For example, iron is used for the transport (hemoglobin, leghemoglobin) and storage (myoglobin) of oxygen (Godfrey *et al.*, 1975), and in several enzymes of the electron transport system, such as cytochromes, cytochrome oxidase (Slater, 1987), ferrodoxins (Matsubara *et al.*, 1987) and flavoproteins (Neilands, 1974). It is also an essential component of several other vital enzymes, such as ribonucleotide reductase (Thelander & Reichard, 1979), nitrogenase (Robson & Postgate, 1980), aconitase, hydrogenases (Stam *et al.*, 1987) and the enzymes of cellular defense systems against oxygen toxicity, such as catalase and superoxide dismutase (Halliwell & Gutteridge, 1988). Considering all the above facts it is very difficult to imagine a form of life that does not depend upon this precious metal (Emery, 1987). Thus, almost all life forms have come to depend on it for their existence and have had to evolve mechanisms for its assimilation (Neilands, 1981b). Although there are a few species of *Lactobacillus,* which utilize manganese and cobalt as biocatalysts in place of iron (Archibald, 1983), these are by far exceptions to the general rule of iron dependence.

1.2 Iron in ecological and cellular environments

The aqueous chemistry of the two most biologically common oxidation states of iron is dictated by their solubility in an environment fixed at neutral pH. At low pH aqueous environments, Fe(II) and Fe(III) exist in the form of soluble $Fe(H_2O)^{+2}$ and $Fe(H_2O)^{+3}$ ions. However, as the pH increases, these ions tend to hydrolyze resulting in the formation of insoluble hydroxy compounds (Reed, 1982; Spiro & Saltman, 1974). At pH 7.0, Fe(II) is quite soluble and one can obtain a 100 mM solution (Hay, 1984). On the other hand, the solubility product of Fe(III) may be as low as 10^{-38} M, which limits the amount of free ferric ion that can be dissolved in water at pH 7.4 to about 10^{-18} M (Schwyn and Neilands, 1987). Moreover, hydroxides of Fe(III) tend to polymerize in solution forming high molecular mass colloids (Spiro & Saltman, 1969).

On average, soil contains approximately 5% iron. However, the availability of iron stands in sharp contrast to its abundance (Neilands, 1981b). For example, sources of iron in aerobic and microaerobic soils are abundant, but only as insoluble minerals. As mentioned above, both Fe(II) and Fe(III) exhibit an exceedingly high affinity for hydroxy ions, with which they form extremely insoluble and stable oxyhydroxide polymers of the general composition FeOOH (for example goethite, hematite, limonite).

Intracellular environments also restrict the availability of iron since it is invariably coordinated by proteins (Critchton & Charloteaux-Wauters, 1987). The majority of the iron is in a chelated or organically bound form, most frequently chelated by the glycoproteins transferrin or lactoferrin, which renders the effective free iron concentration to be on the order of 10^{-18} M (Griffiths *et al.*, 1988). Hence, there is very little "free" iron available to pathogens in human or animal serum.

1.3 Iron in microorganisms: nutritious and noxious

Free Fe(III) in aerobic aqueous environment is limited to an equilibrium concentration of approximately 10^{-18} M, a value far below that required for the optimal growth of microbes (10^{-8} to 10^{-6} M) (Neilands, 1981b). Therefore, microorganisms must have efficient uptake systems for this extremely insoluble critical metal, since biomembranes are impermeable to iron-proteins, polymeric forms of iron, certain iron chelates and iron containing minerals (Nikaido & Vaara, 1985; Spiro & Saltman, 1969). In addition to these problems, microorganisms also have to regulate the process of iron assimilation, since free Fe^{+2} and Fe^{+3} have the potential to cause cellular damage. Iron can act catalytically to generate hydroxyl radicals which are the most potent oxidizing agents known (Fridovich, 1978; Gutteridge, 1987).

$$H_2O_2 + Fe^{+2} \longrightarrow Fe^{+3} + OH^- + OH^\bullet$$

Hydroxyl radicals ($OH^\bullet$) generated by the above Fenton reaction (Dunford, 1987) can react with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA bases, organic acids, etc. When Fe^{+2} concentration is limiting, the Fe^{+3} reduction required for the continued formation of hydroxyl radicals is carried out by the following reaction:

$$O_2^- + Fe^{+3} \longrightarrow Fe^{+2} + O_2$$

This is known as the iron catalyzed Haber-Weiss reaction (Aruoma & Halliwell, 1987; Matzanke, 1991).

Considering the insolubility and toxicity of iron in the presence of oxygen, it is not surprising that many aerobic organisms have evolved a class of ubiquitous iron-storage proteins, the ferritins, which enables them to sequester iron atoms in a non-toxic, yet bioavailable form (Briat, 1992; Grossman *et al.*, 1992). To date, ferritin is known to be manufactured by many mammals, microorganisms (bacterioferritin), fungi, and invertebrates (Mielczarek *et al.*, 1989). Ferritin can accomodate up to 4000 Fe(III) atoms in its mineral core (Harrison & Lilley, 1989). Iron from ferritin can be released in the Fe^{+2} form after reduction of Fe(III) within the protein (Harrison & Lilley, 1989). In this way, ferritins slow or control the metabolic flow of Fe^{+2} and compensate for the lack of an iron excretion mechanism. In other words, ferritin appears to serve as a form of natural insurance against iron toxicity as well as iron starvation.

1.4 Iron levels in microorganisms

Optimum iron levels vary depending on the type of the organism and the environment in which it lives. For example, lactobacilli apparently do not require iron (Archibald, 1983; Neilands, 1981b); on the other hand, magnetotactic bacteria can accumulate up to 1.5% of their dry weight as magnetite (Fe_3O_4) (Frankel *et al.*, 1979). Most bacteria however, contain less than 0.1% iron by dry weight (Lankford, 1973; Neilands, 1974). The iron requirement for the optimum growth of enteric bacteria is about 0.36 μM, while for strict aerobes it is about 1.6 μM (Lankford, 1973; Waring & Werkman, 1942). For fungi and Gram positive organisms, it ranges from 0.4 to 4.0 μM (Weinberg, 1974). These values are dictated by culture conditions; for example, the manipulation of carbon and nitrogen sources can force microorganisms to use alternative metabolic routes which in turn causes higher iron demands (Meyer & Abdallah, 1978; Neilands, 1984b; Subramanian *et al.*, 1968). For a given organism therefore, the concentration of iron required for optimum growth may vary, depending upon the form of iron present in the growth medium and on the mode of iron assimilation used (Pollack *et al.*, 1970). Generally, concentrations up to 10 μM are considered iron sufficient while 0.1 μM is considered to be iron limiting (Neilands, 1984a).

1.5 Iron assimilation in microorganisms

In order to evade iron toxicity, iron homeostasis is strictly regulated and results from a coordinated integration of assimilation, utilization and storage. Iron uptake obviously needs to be regulated in response to variations in environmental iron concentrations, and

has been actively studied for the last fifteen years (Briat, 1992). In general, there are two main iron uptake systems utilized by bacteria, a low affinity and a high affinity system.

1.5.1 Low affinity iron uptake

The low affinity system appears to be non-specific and requires no carrier molecules. Insoluble Fe(III) polymers can bind to the surface of microbial cells and the polymers may then be dissolved through the release of carboxylic acids such as citrate and malate (Winkelmann, 1979). Alternatively, some surface atoms of Fe(III) oxyhydroxide polymers may be less firmly bound, and hence available to the cell. The system has been designated as "low affinity" because relatively high levels of iron are required to achieve optimal bacterial growth rates. Little is known about this system since the evidence for its presence is indirect: organisms that have lost their high-affinity iron uptake systems can still grow in minimal media (Pollack & Neilands, 1970).

1.5.2 High affinity iron uptake

High affinity iron uptake is comprised of two parts, the siderophore (iron chelator) and the cognate transport apparatus. Siderophores are defined as low molecular weight (up to 1500 Da) ligands specific to ferric ions, the biosynthesis of which is tightly regulated by iron (Neilands, 1981a). The term iron-deficient designates, to a microbiologist, a growth environment so devoid of soluble iron that the cell initiates the synthesis of a siderophore(s) and its specific transport system. The ferri-siderophore complex is taken into the cell and the metal ion is liberated either by enzymatic reduction of the iron, or ligand destruction, or by other agents which exhibit higher affinities for ferric ion than the transporting siderophore (Neilands, 1981a).

In microbes as well as in plants and animals, the assimilation of iron is regulated at the membrane transport level, since no biological mechanism exists for the excretion of this essential element (Neilands, 1981a). Each ferri-siderophore complex exhibits a unique chirality around the Fe:ligand centre, which in turn determines the recognition and uptake of the complex by the specific receptor of individual bacterial species.

Microorganisms have thus taken advantage of characteristic steriochemistry within the siderophore ligand in an attempt to solubilize and monopolize environmental Fe(III).

1.6 Siderophores

1.6.1 Microbial distribution of siderophores

Siderophores are commonly produced by most aerobic and facultative anaerobic microorganisms including; phytopathogens (Leong & Neilands, 1982; Enard *et al.*, 1988; Holzberg & Artis, 1983), animal and human pathogens (Chart & Trust, 1983; Griffith *et al.*, 1988), nitrogen fixers (Knosp *et al.*, 1984; Fekete *et al.*, 1989), fungi (Neilands, 1981a; Winkelmann & Huschka *et al.*, 1987), actinomycetes (Hider, 1984) and cyanobacteria (Simpson & Neilands, 1975). Strict anaerobes inhabit areas which have low redox potentials where Fe^{+2} is relatively soluble (Spiro & Saltman, 1969) and thus they have no apparent need for siderophores.

1.6.2 Ferrisiderophore complexes

A ferri-siderophore is a complex ion and the bonding within this complex is considered to be an electrostatic attraction between the positively charged nucleus (Fe^{+3}) and electrons in the ligands. Since electrostatic binding forces between the metal ion and ligands are larger for a higher cation charge density, there is a general trend that Fe^{+3} is less kinetically labile than Fe^{+2} (Emery, 1987).

Depending on the number of ligands available, siderophores can be divided into low affinity chelators which have unidentate, bidentate or tetradentate coordination of iron, and higher affinity chelators, which have hexadentate coordination of iron. A unidentate ligand is one that has a single pair of electrons for donation to a central metal ion. It is only attached at a single position in the coordination sphere and hence does not form a very stable complex ion. On the other hand, a hexadentate ligand donates six pairs of electrons to the central Fe^{+3} ion, thus satisfying the preferred hexacoordinate geometry of Fe^{+3} and is therefore very stable (Matzanke, 1991). Low affinity chelators are often reducing agents or acids which destabilize and solubilize iron from natural sources, whereas high affinity chelators are chiefly the scavengers of scarcely available or organically bound Fe(III).

Both Fe(II) and Fe(III) tend to form six-coordinate octahedral complexes and the atoms donating electrons to the central metal ion may be oxygen, nitrogen or sulfur. With only oxygen around the iron, the redox potential is low, while the potential is high with only nitrogen in the coordination sphere. The ferric ion is usually chelated by up to six atoms, mostly oxygen -for example, in phosphates, phenols, diketones and certain sugars (Hider, 1984; Raymond *et al.*, 1984; Spiro & Saltman, 1974). On the other hand, Fe(II) is

preferentially chelated by nitrogen ligand systems such as 2,2'-dipyridyl and o-phenanthroline (Spiro & Saltman, 1974).

Depending on the participating chelating group, siderophores have been further classified into three classes (Fig 1-1): the catecholates, the hydroxamates and the mixed ligands. All of these are mainly bacterial in origin; however, hydroxamates are also synthesized by fungi. Among the catecholates, the best characterized example is enterobactin, which is a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine. The best studied hydroxamates are the ferrioxamines which are secreted by the *Actinomycetes* (Lankford, 1973) and aerobactin which is secreted by some enteric bacteria (Neilands, 1984a). Examples of the mixed ligands-type are the pseudobactins (Teintze *et al.*, 1981; Buyer *et al.*, 1986), pyoverdines (Wendenbaum *et al.*, 1983; Demange *et al.*, 1987), azotobactins (Demange *et al.*, 1987) and azoverdins (Bernardini *et al.*, 1996) produced by *Pseudomonas* spp., *Azotobacter vinelandii* and *Azomonas macrocytogenes*, respectively.

At non-acid pH, a catechol ligand is a much stronger chelator of iron than a hydroxamate ligand, but since a hydroxamate forms uncharged complexes with iron it is relatively much more stable. Metal catechol complexes are able to undergo intramolecular electron transfer reactions. So the redox state of iron coordinated to catechol is dependent on the pH of the solution and can be repeatedly cycled between Fe(III) and Fe(II) by manipulation of pH (Mielczarek *et al.*, 1989).

1.6.3 Siderophore mediated iron transport

Three different mechanisms may operate in microorganisms for siderophore-mediated Fe transport across cell membranes (Crowley *et al.*, 1991) (Fig 1-2). These mechanisms are presumed to involve membrane-spanning proteins that bind the substrate and undergo a conformational change resulting in iron transport into the cell. However, each of these mechanisms differs with respect to the location of Fe release. The first mechanism (A) involves a transport protein or a permease for intact ferri-siderophore uptake. After transport, the Fe may be removed from the siderophore by a cytoplasmic reductase that reduces the Fe^{+3} and promotes dissociation of Fe^{+2} from the unstable ferrous-siderophore complex (Arceneaux, 1983; Emery, 1987). The desferri-siderophore may then be degraded (non-recycling, as with ferrichrome of *Escherichia coli*) (Hartman & Braun, 1980) or else be secreted back into the environment (recycling, as with schizokinen in *Bacillus*) (Arceneaux *et al.*, 1973). Alternatively, Fe^{+3} may be removed non-reductively

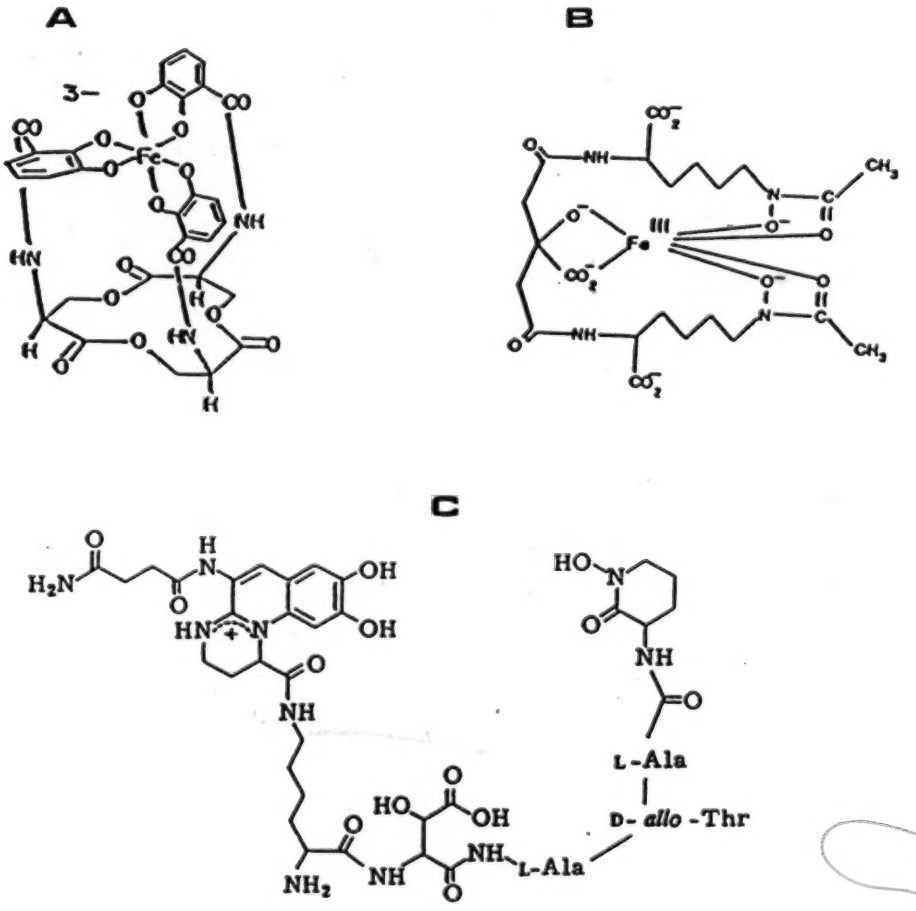

Fig. 1-1 Representatives of the three classes of siderophores based on the participating chelating group. (A) a catecholate, enterobactin. (B) a hydroxamate, aerobactin and (C) a mixed ligand type, parabactin. Adapted from Bagg & Neilands, 1987 (B); and Winkelmann, 1986 (C).

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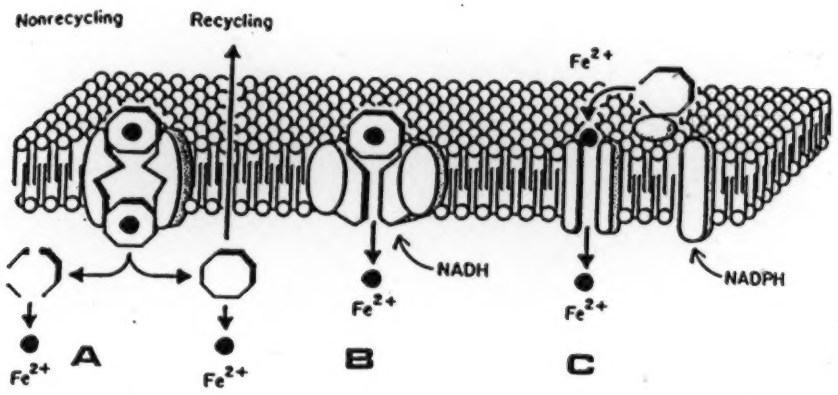

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Fig. 1-2 Generalized mechanism of ferri-siderophore transport by microbes. (A) Ferrisiderophore is transported and Fe is removed in the cytoplasm. Then the deferrated siderophore is either recycled or is destroyed. (B) Direct shuttle with Fe removal at the cell surface without concurrent transport of the siderophore. (C) Indirect shuttle with iron removal at a site remote from the ion channel. Adapted from Crowley *et al.*, 1991.

by hydrolytic destruction of the chelate, as is observed with enterobactin of *E. coli* (Earhart, 1987). Mechanism B, termed the direct shuttle (or the taxi) involves ferri-siderophore binding to a cell surface receptor where Fe is transported into the cell, but this is uncoupled with the transport of the ligand (Emery, 1987; Hider, 1984; Müller *et al.*, 1985; Ratledge, 1987). This may involve a reductive process that transfers Fe^{+2} to a carrier protein (Emery, 1987) or conversely, a non-reductive process with direct ligand exchange of Fe^{+3} to a carrier (Carrano and Raymond, 1978). In the third mechanism (C), Fe is acquired by an indirect shuttle (extracellular dissociation) in which the reductive removal of Fe occurs at a site some distance from the carrier protein. This is perhaps the least efficient mechanism since Fe release is not directly coupled with its transport (Müller *et al.*, 1985). After reduction, the dissociated Fe^{+2} may re-oxidize and precipitate on the cell surface, or may diffuse to the low affinity ion channel/carrier protein for inorganic iron transport.

1.7 Iron and bacterial virulence

The ability of an invading pathogen to multiply successfully under conditions found in its host is essential in establishing an infection. The pathogen must produce the full complement of virulence determinants required for pathogenicity (Griffiths, 1991). Although there is a considerable amount of iron present in the body fluids of humans and animals, as mentioned before, the amount of free iron available to invading bacteria is extremely small. The nutritional shift to a low-iron environment is an important environmental signal for bacteria that have entered a host and they respond by increasing the expression of iron acquisition systems (siderophore apparatus) as well as other Fe-regulated virulence determinants (Neilands, 1981b & 1982; Payne, 1988 & 1993).

A number of studies have shown enhanced resistance to infection in animals in which the levels of iron in serum have been reduced by an iron-deficient diet. This enhanced resistance to infection is reversed if iron-deficient mice are injected with sufficient iron to restore normal levels of iron in the serum (Puschmann & Ganzoni, 1977; Hart *et al.*, 1982). In both animal models and humans, it has been shown that bacterial or viral infections cause up-regulation of the iron-withholding defense system. This system can include: (i) stationing of powerful iron-binding proteins like transferrin, lactoferrin, ferritin at potential sites of invasion, (ii) lowering iron in body fluids and diseased tissues during invasion by synthesis of additional ferritin, and (iii) withdrawing non-heme iron from invaded host cells. These invasion-associated, iron-withholding processes lead to a reduction in serum iron (hypoferremia) and saturation of transferrin with iron and an

increase in serum ferritin (hyperferritinemia) (Weinberg, 1996). For example, in the late stages of AIDS infection, it has been shown that humans become hypoferremic and hyperferritinemic, and they withhold large amounts of iron in bone marrow, brain white matter, muscle, and liver (Boelaert *et al.*, 1996). These symptoms apparently result from the patient's exposure to numerous onslaughts of opportunistic bacterial, fungal and protozoan pathogens (Blumberg *et al.*, 1984; Boelaert *et al.*, 1996).

1.8 Iron uptake in *Escherichia coli*

The ease of generation and isolation of mutants of *E. coli* has resulted in this organism being the best studied in an effort to understand the mechanism of action of the iron transport process. Under iron-deficient conditions *E. coli* synthesizes two different siderophores (Fig 1-3), the main one being the catecholate enterobactin (O'Brien & Gibson, 1970) while the hydroxamate aerobactin (Braun *et al.*, 1987) is produced only by some strains. Enterobactin is a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine (DHBS). It forms highly stable octahedral complexes with ferric ion ($K_f=10^{52}$) and is regarded as the natural iron transport molecule of all *Enterobacteriaceae* (Neilands, 1984a).

There are separate transport systems for enterobactin and for aerobactin. Other hydroxamate siderophores including, coprogen, rhodotorulic acid, and certain ferrichromes and ferrioxamines produced by other bacteria and fungi may also be transported on the aerobactin transport system (Crowley *et al.*, 1991). In addition, there are two Fe-transport systems that are not mediated by siderophores: one for ferric-dicitrate which is inducible under low Fe conditions in the presence of 0.1mM citrate (Frost and Rosenberg, 1973) and a second inducible system for transport of Fe^{+2} (Hantke 1987).

1.8.1 Enterobactin: biosynthesis and transport

The enterobactin synthesis and transport system requires at least sixteen genes: seven for the production of the siderophore, eight for the ferri-enterobactin transport proteins on the outer envelope, periplasm, and cytoplasmic membrane, and one for a protein that removes Fe from the siderophore in the cell cytoplasm.

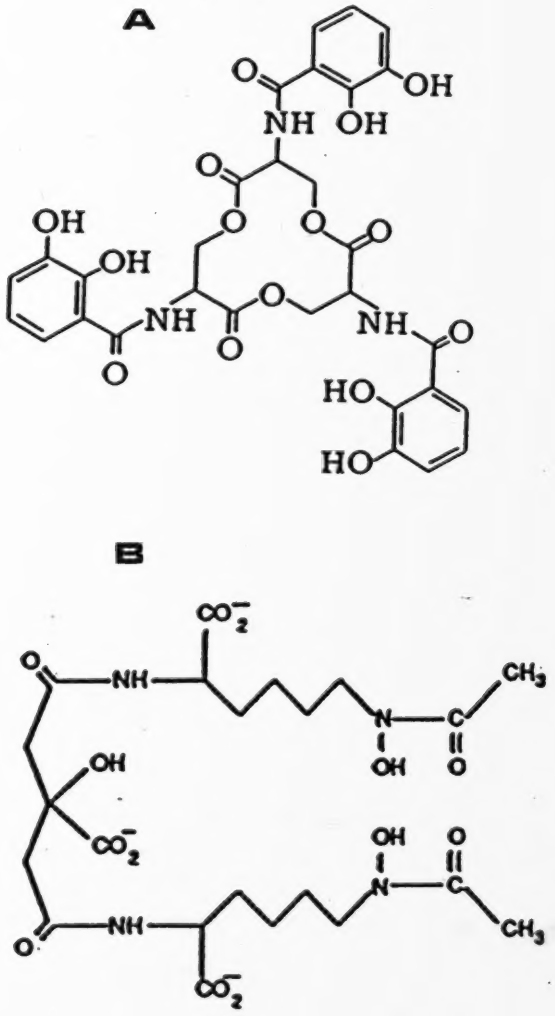

Fig. 1-3 Two main siderophores of *Escherichia coli*. (A) Enterobactin, a cyclic trimer of 2,3-dihydroxy benzoyl serine; (B) Aerobactin, a hydroxamic acid consisting of two residues of acetyl hydroxylysine connected via peptides bonds to the terminal carboxyl groups of citric acid. Adapted from Winkelmann , 1986 (A); and Bagg & Neilands, 1987b (B).

The enterobactin cycle starts with the synthesis of 2,3-dihydroxybenzoic acid from chorismic acid, a general intermediate in aromatic compound biosynthesis (Young *et al.*, 1967; 1971; Young & Gibson, 1969), by three gene products EntC, EntB, and EntA functioning separately in successive reactions (Young *et al.*, 1971) (Fig. 1-4). A complex of four gene products, EntD, EntE, EntF, and EntG functions next, to carry out a series of reactions with enzyme-bound intermediates (Luke & Gibson, 1971). In the end, enterobactin is released from the protein complex.

Once outside the cell, enterobactin binds Fe^{+3}. Approximately eight gene products, including six genes of the *fep* gene cluster, are involved in the movement of Fe^{+3}-enterobactin through the outer membrane and the cell membrane (Cox *et al.*, 1970; Langman *et al.*, 1972). The first gene product of the *fep* gene cluster to encounter the Fe^{+3}-enterobactin is the outer membrane protein FepA. There is indirect genetic evidence that the N-terminal end of FepA is in contact with TonB, an inner membrane protein (Hantke & Braun, 1978; Gunter & Braun, 1990). This association "transduces" the potential energy of the cytoplasmic membrane to drive the energy-dependent transport of the ferri-siderophore across the outer membrane. ExbB, another inner membrane protein, functions to stabilize or activate TonB. Next comes the *fepB* gene product, a periplasmic Fe^{+3}-enterobactin-binding protein. FepD and FepG have been characterized as inner membrane proteins, and FepC has been characterized as a membrane-associated ATP-binding protein. The cells become bright red as Fe^{+3}-enterobactin is accumulated, but remain iron-starved in the absence of the gene product Fes (O'Brien *et al.*, 1971; Langman *et al.*, 1972). Fes cleaves the ester backbone of enterobactin and reduces the Fe^{+3} to Fe^{+2}, thereby increasing the dissociation constant from 10^{-52} M to about 10^{-8} M. Finally, Fe^{+2} is incorporated by ferrochelatase into heme and non-heme iron proteins (Langman *et al.*, 1972).

1.8.2 Aerobactin: biosynthesis and transport

Aerobactin is a hydroxamic acid consisting of two residues of ε-N-acetyl-ε-N-hydroxylysine connected via peptide bonds to the terminal carboxyl groups of citric acid (Warner *et al.*, 1981). Aerobactin is a very important virulence factor, particularly in invasive diseases, as it has the ability to extract iron from transferrin very effectively (Williams, 1979; Williams & Warner, 1980; Konopka & Neilands, 1982).

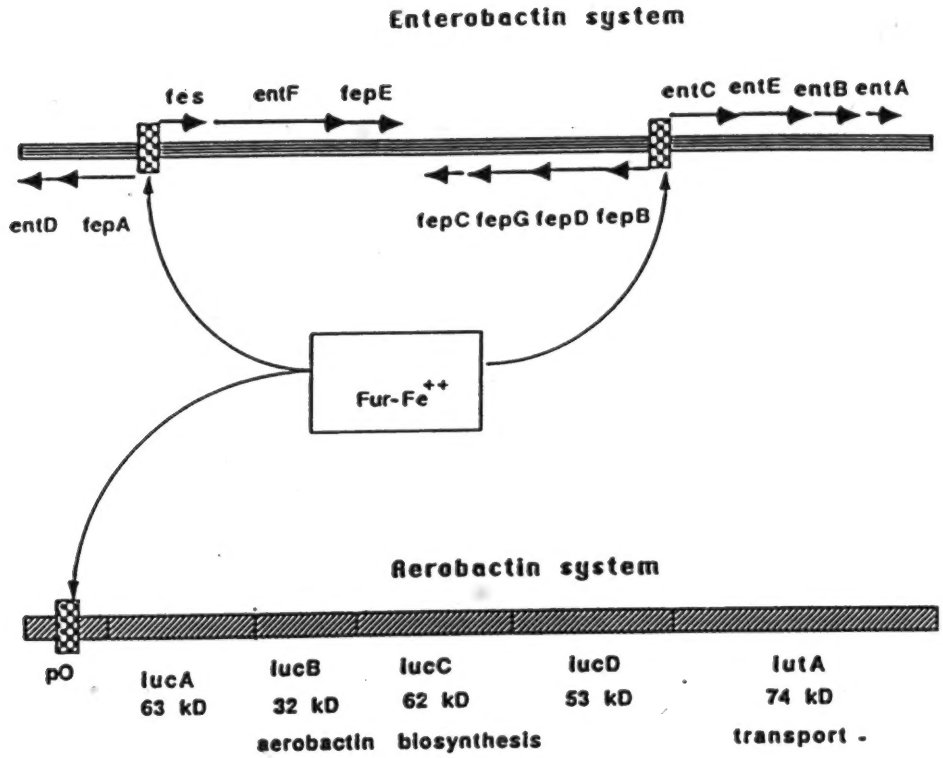

Fig. 1-4 Schematic representation of the organization of enterobactin and aerobactin operons and sites of interaction of the Fur-Fe^{+2} complexes. Symbol : ▩ iron boxes. (Adapted from Crosa, 1989).

The gene products IucD, IucB, IucC and IucA are involved in the actual biosynthesis of aerobactin (Fig 1-4) and a single gene product IutA is involved in transporting the ferri-aerobactin across the outer membrane (de Lorenzo *et al.*, 1986; de Lorenzo & Neilands, 1986). Ferri-aerobactin is then imported into the cell via the ferric hydroxamate uptake proteins FhuD (periplasmic), FhuB and FhuC (both inner membrane) (Braun *et al.*, 1987). The movement of the ferric-aerobactin across the outer membrane is also TonB and ExbB dependent. All of these genes are on the bacterial chromosome, with the exception of those required for the synthesis and transport of aerobactin, which are also carried on pColV plasmids (Valvano & Crosa, 1984; Braun *et al.*, 1987).

1.9 Fur: the master regulator of iron uptake

In *E.coli*, all known siderophore systems are negatively regulated by an Fe-binding protein designated Fur, for ferric uptake regulation (Bagg and Neilands, 1987a; Ernst *et al.*, 1978). Of the 16 genes required for the enterobactin system, 13 genes are assembled into 4 transcriptional units, each starting with a "Fur-box" and regulated by the Fur repressor (Fig 1-4).

Of all the Fur-responding operons, the plasmid-borne aerobactin system in pColV30 is the best understood (de Lorenzo *et al.*, 1987; de Lorenzo *et al.*, 1986). At low intracellular concentrations of Fe^{+2}, the Fur protein has a weak affinity for the operator region and RNA polymerase can easily carry out transcription from the aerobactin promoter. At higher internal concentrations of Fe^{+2}, the Fur protein binds tightly to the operator region and transcription from the aerobactin promoter is blocked (Fig 1-5).

Regulation of Fur-governed systems requires the presence of a 19 bp "iron-box" or "Fur-box" operator consensus motif. Among these 19 bases, the position of 6 bases are highly conserved, three in each half of the dyad (positions 4, 5, 6 and 14, 15, 16; Table1-1). The iron box typically overlaps the -35 or the -10 regions of the promoter (de Lorenzo *et al.*, 1988b).

In addition to Fe^{+2}, several other divalent cations (Co^{+2}, Mn^{+2}, Cd^{+2}, Cu^{+2} and Zn^{+2}) will bind to Fur *in vitro*. In fact, resistance to manganese has formed the basis for a selection to obtain Fur mutants of several Gram-negative bacteria (Prince *et al.*, 1993 and Hantke, 1987). Binding of Fur to the iron-box has been shown experimentally for several genes, including *fur* itself. In all these *in vitro* assays, Mn^{+2} has been used as a

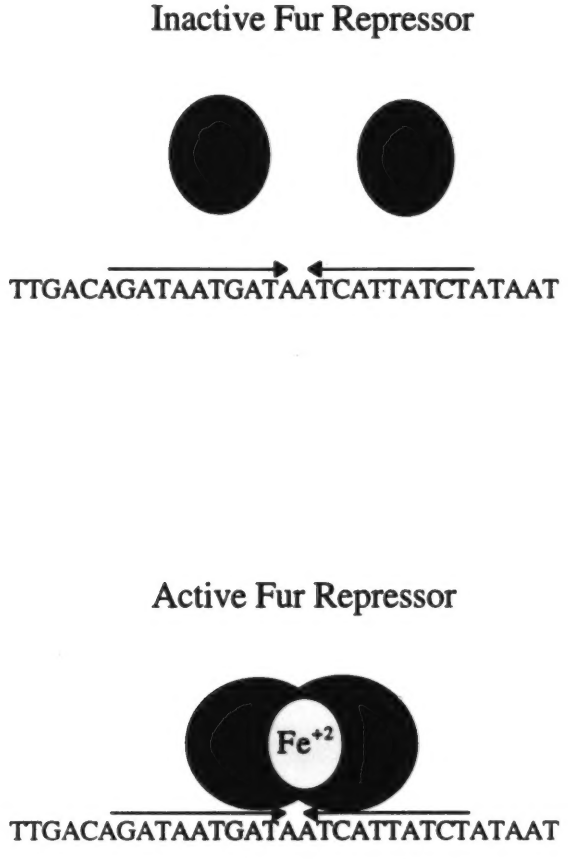

Fig. 1-5 Model showing the interaction of Fur with DNA. In presence of adequate Fe^{+2} the Fur protein is believed to dimerize and form an active repressor. However, in absence of the corepressor, Fur is presumed to exist as a monomer and hence is unable to bind DNA. Based on studies done in *E. coli*, the consensus sequence which is recognized by Fur is a 19 bp palindrome (shown by converging arrows), and has been shown to be present in almost all the genes involved in iron metabolism. [Adapted from Litwin & Calderwood, 1993]

Position	Frequency	Base
1	0.5; 0.2; 0.15; 0.15	G; T; C; A
2	0.6; 0.15; 0.12; 0.13	A; T; C; G
3	0.6; 0.35; 0.05	T; A; C
4	0.82; 0.18	A; T
5	0.95; 0.05	A; T
6	0.85; 0.08; 0.7	T; A; C
7	0.62; 0.2; 0.1; 0.08	G; A; C; T
8	0.8; 0.12; 0.08	A; G; C
9	0.6; 0.2; 0.2	T; A; G
10	0.7; 0.3	A; T
11	0.76; 0.1; 0.09; 0.5	A; T; C; G
12	0.7; 0.2; 0.1	T; C; G
13	0.6; 0.2; 0.1, 0.1	C; T; A; G
14	0.9; 0.1	A; G
15	0.9; 0.07; 0.03	T; A; C
16	0.95; 0.05	T; A
17	0.5; 0.4; 0.1	A; T; G
18	0.8; 0.15; 0.05	T; G; C
19	0.7; 0.2; 0.1	C; T; G

Table 1-1 Frequency of the occurrence of bases at each position in the Fur-box. Data was collected from iron-box sequences of 33 genes. Base #1 is at the 5' end. Adapted from Stojilkovic *et al.*, 1994.

corepressor since Fe^{+2} is unstable and is readily oxidized (Bagg & Neilands, 1987a).

The *fur* locus was first described in *Salmonella typhimurium* (Ernst *et al.*, 1978), but has been best characterized in *E. coli*. Recently, *fur*-like genes have been cloned and sequenced from several organisms including *Pseudomonas aeruginosa* (Prince *et al.*, 1993), *Pseudomonas putida* (Venturi *et al.*, 1995), *Neisseria gonnorhoea* (Berish *et al.*, 1993), *Neisseria meningitidis* (Thomas & Sparling, 1994), *Vibrio cholerae* (Litwin *et al.*, 1992), *Vibrio vulnificus* (Litwin & Calderwood, 1993a), *Vibrio anguillarum* (Tolmasky *et al.*, 1994), *Bordetella pertussis* (Beall & Sanden, 1995), *Haemophilus ducreyi* (Carson *et al.*, 1996), *Yersinia pestis* (Staggs & Perry, 1991), *Legionella pneumophila* (Hickey & Canciotto, 1994), *Campylobacter jejuni* (Wooldridge *et al.*, 1994), and *Klebsiella pneumoniae* (Achenbach & Yang, 1997). All the deduced Fur proteins show a high degree of amino acid similarity.

The *E. coli* Fur protein itself is 148 amino acids in length and 15 to 17-kDa in size. It lacks the consensus "helix turn helix" motif characteristic of many DNA-binding regulatory proteins (Silver & Walderhaug, 1992). DNA footprinting experiments indicate that the Fur complex spirals around the DNA with perhaps more than two Fur units covering three DNA turns. The alpha-helices of the first 82 amino acids of the Fur protein have been implicated in DNA recognition and binding (Saito *et al.*, 1991a & c), whereas the cysteine (Coy *et al.*, 1994) residues present near the middle and carboxy-terminus are believed to be involved in binding the metal ion (Saito *et al*, 1991a & b). There is sufficient proof available in the literature to indicate that the N-terminal domain of the Fur protein possesses has all the information required to recognize the Fur-box, but only the full-sized Fur protein appears to possess a metal binding pocket (Braun *et al.*, 1990;Stojilkovic & Hantke, 1995). There are two metal binding sites per Fur monomer, but it has been shown that only one of these is involved in corepressor activation (Coy & Neilands, 1991). The question, whether Fur dimerizes upon iron binding, or whether Fur occurs as a dimer independent of metal binding still remains to be answered.

1.9.1 Other possible roles of Fur

To date, Fur-box-like sequences have been found in front of several genes which are not directly related to iron metabolism (Stojilkovic *et al.*, 1994). For example, *E. coli* strains with a mutation in *fur* are unable to utilize specific carbon sources (succinate, fumarate or acetate) for growth in minimal media (Hantke, 1987), suggesting a role of Fur beyond that of regulating siderophore biosynthesis and uptake. Fur has been shown to be

essential for *S. typhimurium* to mount an adapative acid-tolerance response (Foster, 1991). However, a recent study by Hall & Foster (1996) indicates that the role of Fur in acid tolerance extends beyond regulating iron acquisition. Their work shows that the acid-sensing and iron-sensing mechanism of Fur are separable by mutation, hence Fur has been implicated as a pH sensor in addition to its well known role of an iron sensor.

Fur has also been shown to be involved in regulating the expression of the PurR repressor. The repressor PurR is involved in regulating the expression of eight purine nucleotide synthesis operons, consisting of genes involved in pyrimidine metabolism as well as of genes whose products are involved in supplying the cell with carbon units necessary for synthesis of methionine, purine etc. (Wilson *et al.*, 1993; and He *et al.*, 1993). The repression of *purR* transcription by the Fur repressor of such genes may help to fine tune the metabolism of the cell to favorable growth conditions. Under Fe-rich growth conditions, a higher growth rate would be maintained by an elevated expression of the purine and pyrimidine biosynthesis operons.

Fur also seems to have a role in regulating the expression of the MetJ repressor of the methionine biosynthesis genes in *E. coli* (Weissbach & Brot, 1991). Here again, the role of the Fur protein could be to ensure increased methionine biosynthesis under favorable iron-rich growth conditions.

The Fur protein is also implicated to have a major role in regulating the expression of genes involved in the flagellum and chemotaxis regulon in *E. coli*. A functional Fur-box in front of such genes might couple iron status of the cell with chemotaxis and motility. Under iron-rich conditions, Fur would repress the expression of flagella in order to keep the cell in a favorable environment (Stojilkovic *et al.*, 1994).

Furthermore, Fur is no longer considered to be a simple repressor. Several *E. coli* and *S. typhimurium* genes have recently been found to be positively regulated by Fur (Guerinot, 1994). Iron-containing superoxide dismutase (SodB) and ribonucleotide reductase are two examples of *E. coli* proteins positively regulated by Fur. Foster and Hall (1992) discovered that out of 41 *S. typhimurium* proteins whose expression was affected by iron excess, 25% were induced and 75% were repressed.

These new findings lead us to believe that Fur protein is a "master regulator" which could be influencing the expression of a much broader repertoire of genes than merely being a repressor of genes involved in iron uptake.

1.10 Role of iron in biosynthesis of aromatic amino acids

The condensation of phosho-enol pyruvate and erythrose-4-phosphate to form 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) and inorganic phosphate is the first committed step in the biosynthesis of aromatic compounds in bacteria (Srinivasan & Sprinson, 1959). It has been shown that the enzyme DAHP synthase contains approximately one mole of iron per mole of enzyme (McCandliss & Herrmann, 1978). DAHP is converted to chorismate in several steps. Chorismate is the branch point intermediate from which all of the aromatic amino acids and aromatic vitamins are derived (Pittard & Gibson, 1970). Chorismate is also the precursor for the enterobactin siderophore of *E. coli* (Young & Gibson, 1969). Phenylalanine and tyrosine are each synthesized in three steps from chorismate, involving rearrangement to prephenate, aromatization to phenylpyruvate or 4-hydroxyphenylpyruvate, respectively, and finally transamination to yield the amino acid end-products. The bifunctional enzymes catalyzing the first two steps of these terminal pathways are chorismate mutase/prephenate dehydratase and chorismate mutase/prephenate dehydrogenase. Tryptophan is synthesized from chorismate in several steps, the last step being catalyzed by the enzyme tryptophan synthase (Pittard & Gibson, 1970).

It has been shown that *E. coli* cells when grown under low-iron conditions, lead to derepresison of certain enzymes of aromatic amino biosynthetic pathway which includes total DAHP synthase acitivity, tryptophan synthase and prephenate dehydratase activity. Infact, prephenate dehydratase activity was shown to be derepressed about 10-fold under low-iron conditions (McCray & Herrmann, 1976).

1.11 Iron uptake in *Pseudomonas* sp.

P. aeruginosa, a well-studied pseudomonad, is an important opportunistic pathogen of humans and is well adapted to conditions of low iron availability imposed by the host. It produces two siderophores, namely the mixed ligand-type pyoverdine and the hydroxamate pyochelin (Fig. 1-6). This species can also utilize a limited number of heterologous ferri-siderophores. Other *Pseudomonas* strains such as *Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas syringae* also produce pyoverdines, but in each case, the composition of the peptide arm differs. As a result, uptake of ferri-pyoverdine is usually strain-specific. Conversely, the chromophore (Fig. 1-6) which is a quinoline derivative, appears to be highly conserved among all the pyoverdines characterized to date. Pyochelin exhibits a relatively low affinity for iron *in vitro*. In contrast, pyoverdine

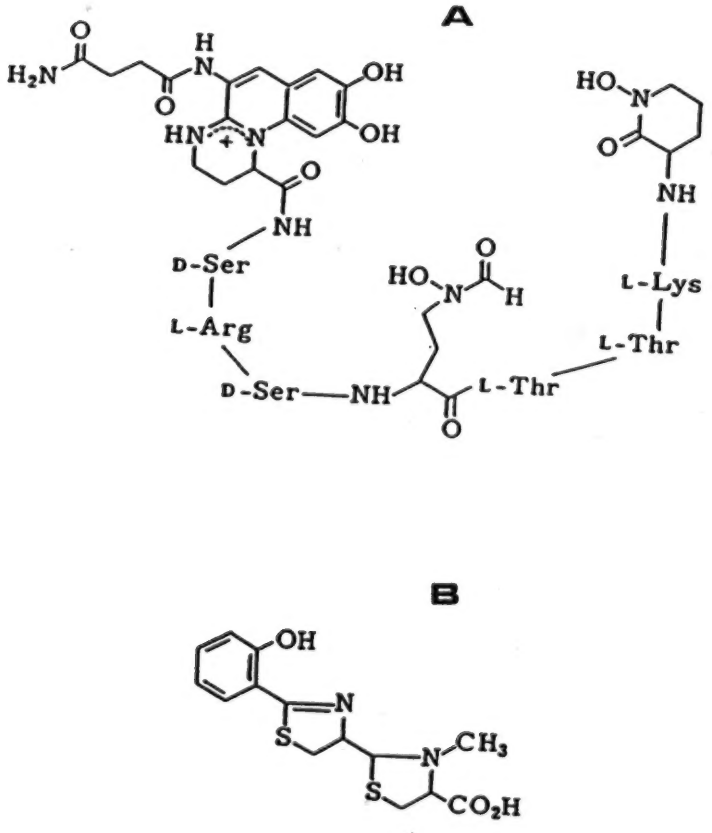

Fig. 1-6 Two main siderophores produced by *Pseudomonas aeruginosa*.
(A) pyoverdine (chromophore + a peptide arm of 6-10 amino acids).
(B) Pyochelin, a hydroxy-phenyl-thiazonyl-methylthiazolidine-carboxylic acid. Adapted from Winkelmann, 1986.

exhibits a markedly higher affinity for iron and is capable of removing transferrin-bound iron *in vitro*. The binding constant of iron for pyoverdine (10^{32}) is significantly greater than that of pyochelin (10^{5}) at acid pH (Wendenbaum *et al.*, 1983). As figure 1-6 shows, pyoverdine is composed of a 6,7-dihydroxyquinoline-containing yellow green fluorescent chromophore joined to the N-terminus of a partly cyclic octapeptide{D-Serine-L-Arginine-D- Ser- L- N^5 OH - Ornithine - L- Lysine -L - N^5 - OH - Orn - L - Threonine - L- Threo}. The hydroxamate groups formed by the two N^5-hydroxyornithine residues participate in the binding of iron together with the catecholate group of the chromophore. Pyoverdine is very water-soluble with a molecular weight of ≈ 1500 Da and binds Fe(III) with a stoichiometry of 1:1 (Gensberg *et al.*, 1992). Recently, some of the genes responsible for the biosynthesis of pyoverdine have been isolated. The product of gene *pvdA* is the enzyme L-ornithineN^5-oxygenase (Visca *et al.*, 1994), which catalyses the formation of the hydroxamate ligands of pyoverdine. The product of another gene *pvdD* has been proposed to be involved in the assembly of the peptidic moiety of the siderophore as a non-ribosomal peptide synthetase (Merriman *et al.*, 1995). The products of *envCD* gene cluster are implicated in pyoverdine secretion and an outer membrane protein encoded by the *fpv* gene functions in ferri-pyoverdine uptake (Leoni *et al.*, 1996). Not much is known, however, about the biosynthesis of the chromophore moiety of pyoverdines. The only information available to date is that the precursor is tyrosine in *P. fluorescence* (Nowak-Thompson & Gould, 1994).

Pyoverdine synthesis, in general, was found to be negatively regulated by the level of available iron. However, recognition sequences for the Fur-repressor were not identified in the control regions of any of the genes responsible for pyoverdine biosynthesis and uptake. Another gene *pvdS* was identified (Cunliffe *et al.*, 1995) which encodes for an alternative sigma factor PvdS and this has been implicated to play a master regulatory role in the activation of pyoverdine biosynthetic genes. The expression of *pvdS* was not auto-regulated, but was found to be negatively regulated by Fur. Thus, under iron-sufficient growth conditions, the Fur repressor would block transcription from the *pvdS* promoter and indirectly prevent the expression of the pyoverdine biosynthetic genes. During iron-starvation, repression is relieved and the alternative sigma factor PvdS is produced, which confers to RNA polymerase specificity for pyoverdine promoters of the biosynthetic genes (Venturi *et al.*, 1995).

Pyochelin is a structurally unique, blue-white fluorescent, phenolate siderophore (Fig. 1-6), which appears to be produced by all strains of *P. aeruginosa*. It is comprised of a salicyl ring bonded to a thiazoline ring which is itself bonded to a N-methylthiazolidine ring. This siderophore is poorly soluble in water and has a low molecular weight (325 Da) (Visca *et al.*, 1992). The stoichiometry of iron binding appears to be two pyochelin molecules to one Fe^{+3} ion. However, pyochelin is extremely active in iron transport and growth stimulation in medium containing transferrin and has been implicated in the pathogenicity of *P. aeruginosa* (Ankenbauer *et al.*, 1988; Wolz *et al.*, 1994). To date, only a few genes responsible for the biosynthesis of pyochelin have been identified. The product of the *pchR* gene was shown to be a positive activator as well as a repressor of pyochelin biosynthesis (Heinrichs & Poole, 1996) and has a functional Fur-box upstream of the gene. Another gene product FptA has been shown to be the outer membrane receptor for ferri-pyochelin transport (Ankenbauer & Quan, 1994).

1.12 *Azotobacter vinelandii*

Azotobacter vinelandii is a Gram-negative bacterium with large ovoid cells 1.5-2.0 µm or more in diameter, pleomorphic and ranging in shape from rod to coccoid. They do not produce endospores, but form cysts, are motile by peritrichous flagella, are obligately aerobic nitrogen fixers, are catalase positive and occur in soil and water. The mol% G+C of their DNA is 64.9-66.5 (Krieg & Holt, 1984). Colonies are not slimy, but variant colony forms may arise due to the quantity of extracellular polysaccharides (alginates) produced. *A. vinelandii* is very similar to flourescent pseudomonads and belongs to the same γ subgroup of the proteobacteria. *A. vinelandii* produces siderophores and becomes naturally competent in Fe-limited and Mo-limited media (Page & von Tigerstrom, 1988; Page, 1985).

1.12.1 Iron regulation in *A. vinelandii*

A. vinelandii is widely known for its ability to fix nitrogen aerobically (Sadoff *et al.*, 1979). An adequate supply of iron is required by this organism to support its extremely high respiration rate, which in turn serves to protect the oxygen-labile nitrogenase system (Robson and Postgate, 1980). Such a high respiratory rate generates toxic oxygen radicals that are destroyed by active catalase and superoxide dismutase (Jurtshuk *et al.*, 1984). These activities are very much dependent on a commensurate supply of iron.

A. vinelandii produces four siderophores (Fig 1-7): one pyoverdine called azotobactin (Demange *et al.*, 1986 and Page *et al.*, 1991) and three catecholates–namely, azotochelin (Page & Huyer, 1984), aminochelin (Page & von Tigerstrom, 1988) and protochelin (Cornish & Page, 1995). In addition to these iron-repressible Fe^{+3} chelators, *A. vinelandii* also produces 2,3-dihydroxybenzoic acid (2,3-DHBA) which is loosely regulated by iron availability and promotes iron solubilization and uptake (Page & Huyer, 1984; and Sevinc & Page, 1992). However, it is known to follow a unique pattern of sequential derepression of its siderophores (Page & Huyer, 1984). At Fe^{+3} concentration of ≤ 7 μM the catechol siderophores are produced coordinately (Page & von Tigerstrom, 1988). If iron continues to be limiting (≤ 3 μM Fe^{+3}), then azotobactin is formed (Page & von Tigerstrom, 1988). When iron is supplied to iron-limited cells, azotobactin is repressed first, followed by the catechols, until 2,3-DHBA remains as the sole ligand (Page & Huyer, 1984). Such a pattern of sequential siderophore regulation is not seen in enteric bacteria (McIntosh & Earhardt, 1977). This suggests that in *A. vinelandii,* iron-repressible genes may be regulated by a mechanism other than that seen for enteric bacteria, or that the affinity of the Fur-Fe^{+2} complex for iron-repressible promoters may be different, or that there could be involvement of some yet unknown factors (Page & Patrick, 1988). So far, the Fur protein has not been detected in *Azotobacter*. This raises questions about the factors–including Fur involvement–influencing iron-regulated transcription in this organism.

1.12.2 Genetics of *A. vinelandii*

A single cell of *A. vinelandii* from a mid-exponential culture contains 1.5×10^{-13} g DNA, a value approximately 40 times that of *E. coli* (Sadoff *et al.*, 1979; Phadnis *et al.*, 1988). However, the size of the *A. vinelandii* chromosome is approximately 4700 kb (Manna & Das, 1993; Maldonado *et al.*, 1994), which is almost the same size as the chromosomes of *E. coli* and *S. typhimurium.* According to simple calculation of DNA per cell, *A. vinelandii* is polyploid and appears to have at least 40 copies of its chromosome/cell. However, Maldonado *et al.* (1992) concluded from observations on reversion rates of transposon-induced mutations, instability of heterozygotic transconjugants and transformants, as well as segregation characteristics of chromosomal *lac* fusions, that *A. vinelandii* cannot be a polyploid bacterium. This controversy was laid

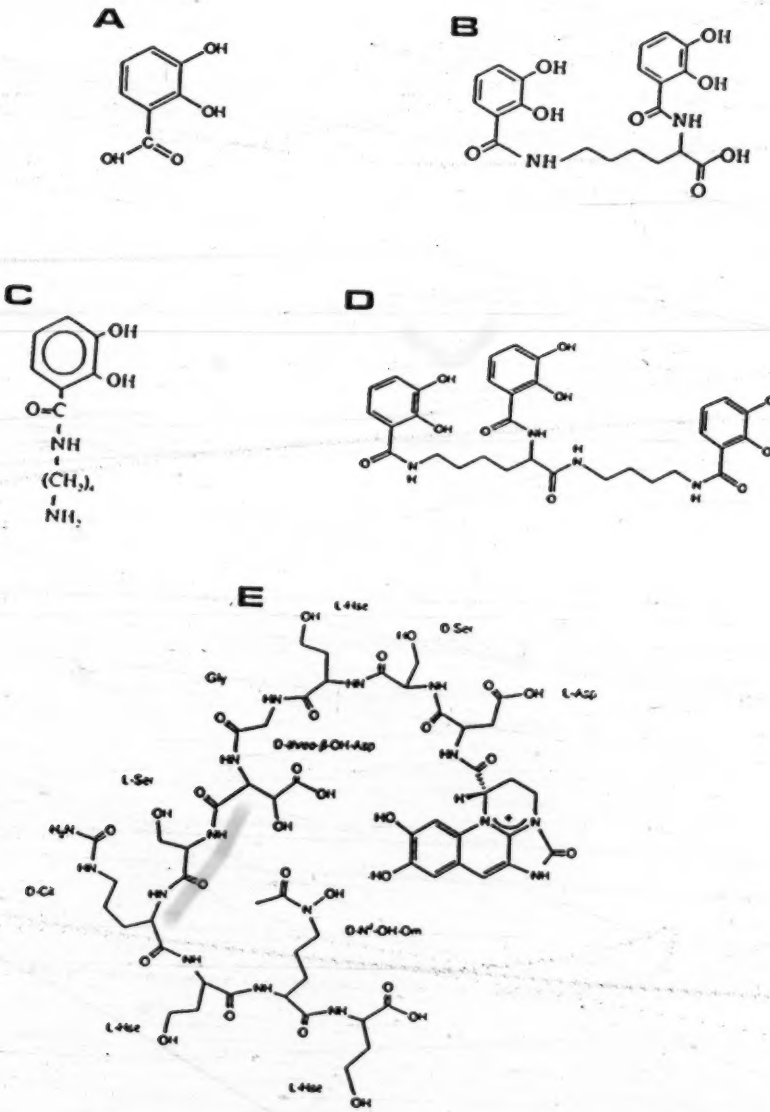

Fig. 1-7 Structures of siderophores secreted by *Azotobacter vinelandii*. (A) 2,3-Dihydroxy benzoic acid; (B) Azotochelin (*N,N'*-bis-(2,3 dihydroxybenzoyl -lysine); (C) Aminochelin (2,3-dihydroxybenzoylputrescine); (D) Protochelin (a tricatecholate, condensation product of B & C); (E) Azotobactin (a pyoverdine, chromophore-aspartyl-homoseryl-seryl-homoseryl-citrullinyl-seryl-glycyl-hydroxyaspartate). Figures adapted from Winkelmann, 1986 (B); Page & von Tigerstrom, 1988 (C); Cornish & Page, 1995 (D); Page *et al.*, 1991 (E).

to rest in 1994 (Maldonado *et al.*) when surveys of DNA content per cell using flow cytometery proved the existence of ploidy changes during the growth cycle in rich medium. Early exponential phase cells have a low ploidy level, but a continuous increase of DNA content per cell is observed during growth. Late exponential phase may contain more than 40 chromosomes per cell, while cells in the early stationary stage may contain about double that number. In late stationary phase cultures, the DNA content per cell is even higher, probably over 100 chromosome equivalents per cell. It has been observed that there is a dramatic change in old stationary phase cultures during encystment, when the population of highly polyploid bacteria segregates cells with low ploidy. Cells with low ploidy are also formed when old stationary phase cultures are diluted into fresh medium (Maldonado *et al.*, 1994). However, we still do not know how many chromosomes are present per cell in this low ploidy stage.

Addition of rifampin to exponential phase cultures causes a rapid increase in DNA content, indicating that *A. vinelandii* initiates multiple rounds of chromosome replication per cell division (Maldonado *et al.*, 1994). The existence of this severe asynchrony between replication and cell division provides an explanation as to why *A. vinelandii* becomes more polyploid than enteric bacteria during rapid growth.

There are other examples in the bacterial kingdom of bacteria having multiple chromosomes. *Desulfovibrio gigas* may have up to 17 copies of its genome per cell and *Desulfovibrio vulgaris* may have up to 4 chromosomes/cell (Postgate *et al.*, 1984). *Micrococcus radiodurans* has been shown to contain between 4 and 11 chromosome equivalents per cell (Hansen, 1988). Existence of multiple chromosomes has also been reported in the cyanobacterium *Synechocystis* sp. (Labarre *et al.*, 1989).

The biological significance of this polyploidy remains a mystery. There is no information available on whether all copies of chromosomes are functioning simultaneously or not. *A. vinelandii* has one of the highest respiratory quotients known (Gutschik, 1980). This suggests the presence of an unusually large amount of respiratory enzymes that may be produced from multiple copies of genes. As a lot is yet to be learned about this peculiar characteristic of *Azotobacter*, this is speculative.

1.12.3 Genetic transformation of *A. vinelandii*

Genetic transformation in a bacterium can be defined as a process in which a bacterial recipient can take up exogenous DNA and incorporate it into its own chromosome by

homologous recombination or convert it into an autonomous extra chromosomal replicon. Four successive steps are required for successful transformation: (i) the development of cell competence, (ii) DNA binding to the competent cell, (iii) the uptake of DNA and its processing, and (iv) phenotypic expression of the new genotype (Smith *et al.*, 1981).

Natural competence is considered different from artificial competence, the latter resulting from physiochemical treatments which force the uptake of the transforming DNA. Natural competence, in the other hand, is a physiological state which promotes transformation (Stewart & Carlson, 1986).

A. vinelandii is one of several eubacteria that can be naturally induced to competence for genetic transformation by chromosomal as well as plasmid DNA. This occurs in Fe-limited and Mo-limited media (Page and von Tigerstrom, 1978; Page, 1985; Doran *et al.*, 1987). The actual mechanisms that enable *A. vinelandii* to bind, envelope, and transport plasmid DNA are not known.

Broad host range plasmids derived from the IncQ plasmid RSF1010 have been shown to be stably maintained in *A. vinelandii* when introduced via conjugation or artificial transformation (Kennedy & Robson, 1983; David *et al.*, 1981). Unlike chromosomal DNA-mediated transformation in *A. vinelandii,* the uptake and establishment of plasmids pRK2501, RSF1010 and pGSS15 were shown to transform *A. vinelandii* at all stages of growth with the same frequency (Glick *et al.*, 1985).

Plasmid DNA-transformed *A. vinelandii* cells have been found to function less efficiently in several ways as compared to cells without the plasmid. They are unable to produce normal levels of siderophores under iron-limiting conditions, they are severely limited in their ability to fix nitrogen and they are approximately one-fifth the size of the non-transformed cells (Glick *et al.*, 1985 & 1986). It is surmised that these impairments are caused by a "metabolic load" imposed by the maintenance of the plasmid in transformed cells. When *A. vinelandii* is transformed with the low copy number, broad host range plasmid pRK290, the consequences of this transformation are less severe than those observed with the high copy number plasmids. This difference is most likely due to the level of expression of foreign plasmid-encoded proteins such as those involved in antibiotic resistance (Glick *et al.*, 1989).

1.12.4 Generation of *Azotobacter* mutants

Mutant phenotypes of *Azotobacter* have been difficult to obtain (Sadoff *et al.*, 1979). Several attempts have been made to obtain mutations in *Azotobacter* genes and the most successful results have been obtained by using transposon mutagenesis (Contreras *et al.*, 1991). Since the organism has unusually high number of chromosome copies per cell, obtaining pure mutants is a challenge. This can be made more explicit by considering an example: when a transposon is inserted in one copy of the *Azotobacter* chromosome, it might inactivate the allele of an essential gene in that chromosome. The cell, however, would continue to grow and divide, since the essential gene product would continue to be formed through the expression of the wild-type alleles located on other identical copies of the chromosome. Due to segregation in the presence of antibiotic, cells with more and more copies of the chromosome containing the mutant alleles would accumulate. Since the segregation is random, it may take many generations before the transposon is present in all copies of the chromosome. If the transposon has inserted in an essential gene, a homokaryon would be fatal, but a heterokaryon should survive (Phadnis *et al.*, 1988).

1.12.5 Generation of *A. vinelandii* mutants defective in siderophore production

A first step in studies of iron regulation in *A. vinelandii* is the generation of mutants defective in iron-regulated gene activity. Transposon mutagenesis, using a derivative of Tn*5* containing a promoterless *luxAB* fusion was used by Sevinc & Page (1992) to generate *A. vinelandii* strains defective in siderophore production.

Tn*5* was delivered on a suicide vector (pTn*5luxAB*), so that Tn*5* would only persist in cells where it had transposed into the chromosome (Berg *et al*, 1989). The transposon, Tn*5luxAB* contains a promoterless luciferase gene fusion adjacent to the left border of the transposon. The left IS*50* was truncated from the outer limit so that, when inserted into its target, a promoter from the host can express the luciferase (*lux*) genes and produce bioluminescence proportional to the strength of the promoter (Sevinc, 1992). Transfer of pTn*5luxAB* into *A. vinelandii* UW generated over 500 Kan^R colonies. Since the plasmid was not maintained in *A. vinelandii*, stable Kan^R strains were assumed to be the result of Tn*5* transposition into the chromosome. In fact, all of the Kan^R strains were stable and did not lose antibiotic resistance or Lux activity, even after transfer under non-selective conditions. After screening the insertion strains for iron-repressible luciferase activity, these strains were categorized into several groups. From those that expressed strong iron-

regulated bioluminescence, several mutants were screened in an attempt to detect siderophore negative mutants. Several interesting phenotypes were found, including strains which did not form catechols (F196) and strains which demonstrated normal catechol formation, but low or relatively unrepressed azotobactin formation. In addition, a strain (D27) that was unable to form azotobactin was also detected.

1.12.6 Biosynthesis of *Azotobacter* siderophores

Little is known about the biosynthesis of *Azotobacter* siderophores. However, the results of the study done by Sevinc & Page (1992) demonstrated several interesting points. A single iron-repressible Tn*5luxAB* insertion in strain F196 inactivated the production of all known catechol siderophores, including 2,3-DHBA. This suggests that the biosynthetic genes for catechol siderophores may be organized in an operon (similar to the case present in enterobactin system) and that the mutation in F196 is polar. Alternatively, the biosynthesis of 2,3-DHBA may be a prerequisite for catechol siderophore synthesis, analogous to the requirement for the *entA*, *entB*, and *entC* gene products in enterobactin biosynthesis (Ozenberger *et al.*, 1989). However, addition of 2,3-DHBA exogenously in the growth medium did not suppress the mutation in strain F196 and promote catechol siderophore biosynthesis. Therefore, our level of understanding of catechol biosynthetic system in *Azotobacter* is still in its infancy.

Azotobactin belongs to a large family of peptidic siderophores collectively called pyoverdines. The biosynthesis of pyoverdine from *Pseudomonas sp.* is slowly being elucidated but very little is known about the synthesis of azotobactin. The only known fact is that the precursor of the chromophore moiety is derived from 3,4-dihydroxyphenylalanine (Fukasawa and Goto, 1973). This is in contrast to the findings in *P. aeruginosa* (Stinzi *et al.*, 1996) and *P. fluorescens* (Nowak-Thompson and Gould, 1994), where it has been shown that the precursor of the chromophore moiety of pyoverdine siderophore is derived from tyrosine. One can only speculate that the peptide arm of azotobactin is synthesized like the pyoverdines via a non-ribosomal peptide synthetase.

1.12.7 Genetic regulation of *A. vinelandii* siderophores

Very little has been published concerning the genetic regulation of siderophore synthesis in *A. vinelandii* siderophores. In the study done by Sevinc & Page (1992), it

was observed that loss of catechol siderophores led to an increase in the level of azotobactin produced. This may reflect the functional role of *A. vinelandii* siderophores. Lower affinity catechol siderophores in higher concentrations appear to be efficient solubilizers of mineral iron sources, while the higher affinity siderophore azotobactin in low concentration appears to be an effective scavenger of soluble iron (Page & Huyer, 1984; Page & von Tigerstrom, 1988). Azotobactin is hyperproduced only when the amount of catechol siderophore produced in iron-limited medium fails to liberate iron from insoluble minerals. Thus, catechol-siderophore-negative cells may sense iron-limitation, although mineral iron is present. Under these conditions, azotobactin is produced, soluble iron is scavenged and iron-limited growth is promoted, resulting in enhanced production of azotobactin (Sevinc & Page, 1992).

Since the catechol-negative and azotobactin-negative mutants can be obtained independently, their synthesis is probably not functionally coupled. However, Glick *et al.* (1988) obtained some conflicting results when they constructed siderophore mutants of *A. vinelandii* using ethyl methanesulfonate. In their study, they isolated some 32 stable non-fluorescent mutants; all of them failed to produce azotobactin and they were also severely impaired in the production of azotochelin. Hence, they believed that the synthesis of azotobactin and azotochelin is functionally coupled. It seems more likely, however that their results can be explained by the selection of mutant strains with multiple mutations.

Many questions remain as to why there is such a unique system of coordinate production of siderophores in *A. vinelandii* and more importantly how this system is regulated at the genetic level.

1.13 Preliminary studies on *A. vinelandii* strain UA22 [1]

During the course of the study of Sevinc & Page (1992), a mutant with iron-repressible bioluminescence was identified. This mutant strain, UA22, seemed to produce lower amounts of azotobactin than the wild type strain UW[2].

Bioluminescence of 1 to 2 day old plate cultures of UA22 was strong in low iron (1 µM Fe^{+3}) medium and only faintly present in high iron (300 µM Fe^{+3}) medium. Siderophore

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[1] Unpublished results of M.V. Woestyne and W.J.Page 1991

[2] The preliminary work to characterize this mutant was done by Dr. Marleen Vande Woestyne, a postdoctoral fellow in the laboratory of Dr. W.J. Page.

production by strain UA22 was repressed in liquid Fe-sufficient (20 μM Fe^{+3}) medium and was derepressed in Fe-limited medium, following the sequential derepression pattern obtained in other studies with the wild type (Page & Huyer, 1984; Page & von Tigerstrom, 1988). The first appearance of bioluminescence in Fe-limited medium was observed at 15 h, at the beginning of the stationary phase of growth and near the onset of azotobactin production.

In an effort to identify the disrupted gene in UA22, genomic DNA from this strain was isolated and screened for the presence of the Tn*5* insert. Purified genomic DNA was partially digested with *Sau*3A and DNA fragments in the 20-25 kb range were cloned into the *Bam*H1 site of the cosmid vector pLAFR3 and transduced into *E. coli* VCS257. Cosmid containing clones (2025 isolates) were selected and screened for Kan^R, indicating the presence of the insertion. Only one clone (pMVW31) was Kan^R and demonstrated Fe-repressible Lux activity in the Petri plate contact printing assay (Sevinc & Page, 1992). To further localize the iron-repressible promoter (IRP) controlling the expression of *luxAB* in pMVW31, several steps of subcloning were done (Fig 1-8). pMVW94 (12.8 kb) was the smallest subclone which was Kan^R as well as Lux^+. Further subcloning of pMVW94 resulted in a 3.2 kb *Sma*I-*Cla*I fragment which, after cloning into pUC119, resulted in the plasmid pMVW97. The plasmid pMVW97 was Lux^+ and Kan^s. From the map of pTn*5luxAB*, it was determined that there was a *Pst*1 site 222 bases from the start of the *lux* genes (unpublished data). Therefore, the IRP should be located in the 1.3 kb *Pst*1-*Sma*1 fragment of pMVW97. This fragment was subcloned into the *Pst*1-*Sma*1 site of pUC119 to construct pMVW98.

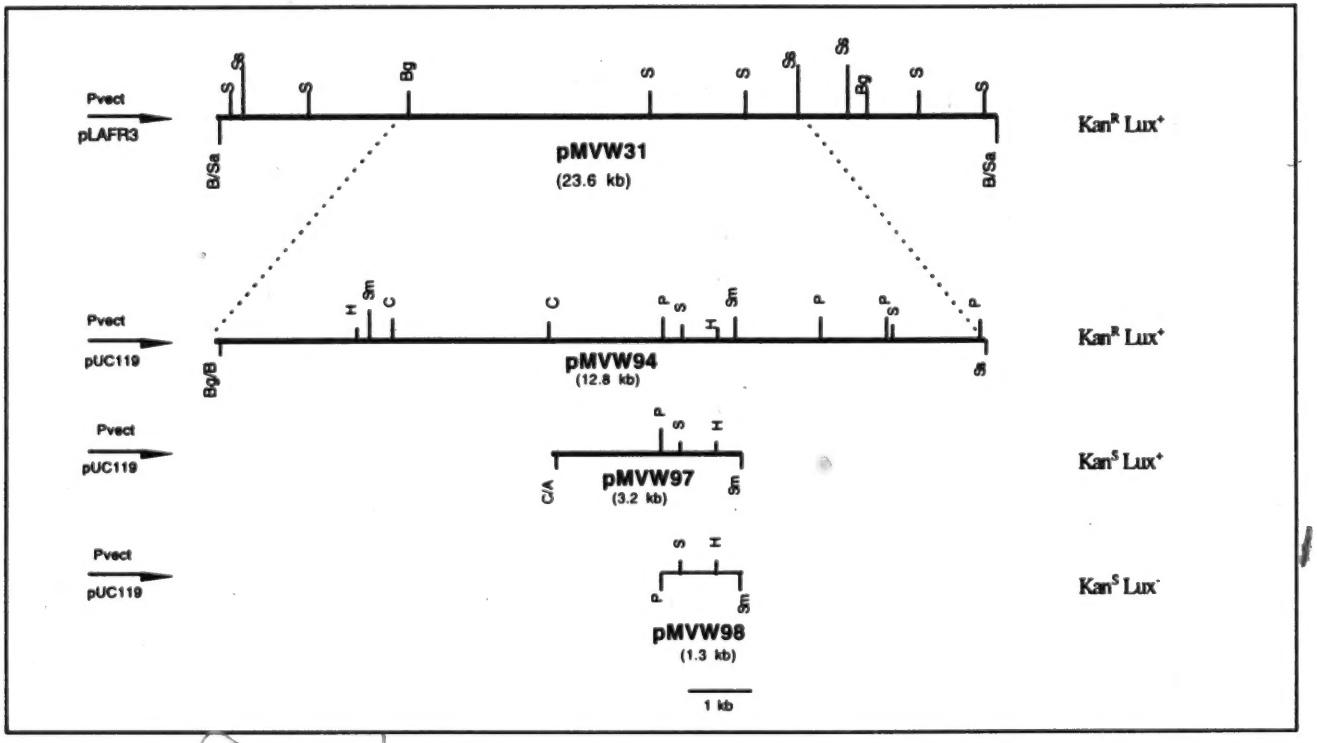

Fig. 1- 8 Figure showing the subcloning strategy used by Dr. M. Vande Woestyne for obtaining pMVW98 from pMVW31. Vector and the orientation of the promoter in the vector is shown. The abbreviations used for the restriction enzyme are: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Ss, *Sst*I; Sm, *Sma*I; Sa, *Sau*3A.

1.14 Thesis Objectives

The aim of the work described in this thesis was to further characterize the mutant strain, UA22, and to carry out further investigations on the subclone pMVW98 isolated by Dr. Marleen Vande Woestyne. We hoped the identification and characterization of the iron-regulated promoter from UA22 would give us an insight into the genetic regulation of iron metabolism in *Azotobacter*. This study reports the characterization of the mutant phenotype and the genotype of the strain UA22, sequencing of the subclone and identification of gene mutated by Tn*5luxAB*. The nature of iron-regulation was studied *in vivo* as well as *in vitro* using Lux and β-galactosidase reporters and *in vitro* using DNA-binding assays. Efforts were made to understand the promoter activity present in the subclone pMVW98 and to locate the start of the transcript by using S1 nuclease and primer extension analysis. An attempt also was made to look for the presence of a Fur-like protein in *A. vinelandii*.

Chapter 2
Materials and Methods

2.1 Bacterial strains

A. vinelandii UA22 was constructed by mutagenesis with pTn*5luxAB* by M. Serdal Sevinc (Department of Microbiology, University of Alberta). A list of *E. coli* and *A. vinelandii* strains used in the present study is given in Table 2-1.

2.2 Media and growth conditions

A. vinelandii strains were grown aerobically at 28 to 30 °C with shaking at 225 rpm in Burk's medium (Page, 1987). Glucose (1%, w/v) was used as the carbon source and the nitrogen source was provided by ammonium acetate (110 mg/100 ml); this medium was called BBGN. For iron-responsive growth studies and for β-galactosidase assays, glassware was first washed with 4 M HCl and then with 50 mM EDTA (pH 7.0), followed by thorough rinsing with de-ionized water to minimize contamination by iron. Iron-limited Burk's medium (OFeBBGN) contained no added iron and soluble iron was varied by the addition of ferric citrate. Insoluble iron, in the form of micaceous hematite, was added to OFeBBGN to enhance siderophore production (Page, 1993; Page & Huyer, 1984). The medium was inoculated and incubated at 30 °C with gyratory shaking at 225 rpm, in a New Brunswick model G-76 water bath shaker (Page, 1987). Burk's medium slants (5 ml) containing 1.5% to 1.8% agar in 16-mm diameter tubes were used to maintain the *A. vinelandii* cultures.

E. coli strains were grown in Luria-Bertani (LB) medium at 37 °C (Ohman, 1988) (per liter: 10 g tryptone, 10 g NaCl, 5 g yeast extract, pH 7.2). Deferrated ethylenediamine-di-(o-hydroxyphenyl) acetic acid (EDDHA), a synthetic Fe^{3+} chelator, was added to LB medium to limit iron availability (Page, 1993). Media were always prepared using de-ionized water from the Milli-Q water purification system (Millipore Corp., Bedford, MA).

Antibiotics were added as appropriate at the following concentrations: kanamycin (Kan; stock solution of 50 mg/ml in water), *E. coli* culture, 50 µg/ml and *A. vinelandii* culture, 7.5 µg/ml; ampicillin (Amp; stock solution of 50 mg/ml in water), *E. coli* 80 µg/ml; chloramphenicol (Chl; stock solution 34 mg/ml in 95% ethanol), *E. coli* 50 µg/ml and *A. vinelandii* 20 µg/ml; tetracycline (Tet; stock solution 5 mg/ml in 98% ethanol), *E. coli* 50 µg/ml and *A. vinelandii* 12.5 µg/ml.

Organism	Relevant genotype / properties	Source	Reference
A. vinelandii			
D13	UW Tn*5luxAB* mutant	W. J. Page	Sevinc & Page, 1992
D48	UW Tn*5luxAB* mutant	W. J. Page	Sevinc & Page, 1992
E21	UW Tn*5luxAB* mutant	W. J. Page	Sevinc & Page, 1992
F196	UW Tn*5luxAB* mutant, catechol negative, azotobactin overproducer	W. J. Page	Sevinc & Page, 1992
UA22	UW Tn*5luxAB* mutant	W. J. Page	Sevinc & Page, 1992
UW	Wild type	W. Brill	ATCC 12837
VK20	*recA* deletion mutant of UW	H. K. Das	Venkatesh *et al.*, 1990
E. coli			
MV1193 F'	Δ(*lac-pro*AB)*rpsL thi endA spc*B15 *hsd*R4 Δ (*srl-rec*A) 306::Tn*10* (tet^r) *lacI*^{q} F'	Lab stocks	Zoller & Smith, 1987
JM106	*end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A1 λ$^-$ Δ(*lac-pro*AB)	M. Farinha	Yanish-Perron *et al.*, 1985
DH5α F'	F' *end*A *hsd*R17 (r_k^- m_k^+) *sup*E44 *thi*-1 *rec*A *gyr*A (Nal^r) *rel*A1 Δ(*lac*ZYA-*arg*F) U169 *deo*R (ϕ80d*lac*Δ(*lac*Z)M15)	Lab stocks	Raleigh *et al.*,1989

Table 2-1 List of *A. vinelandii* and *E. coli* strains used in the study.

and *A. vinelandii* 10 µg/ml; chloramphenicol (Chl; stock solution of 34 mg/ml in 95% ethanol), *E. coli* culture 40 µg/ml and *A. vinelandii* 10 µg/ml.

2.3 Analysis of siderophores in *A. vinelandii* cultures

Cells were centrifuged in 13 mm tubes in a bench top centrifuge (International Equipment Co., Needhan, Massachusetts, USA) at setting #5 for 10 min. The culture supernatant fluid was acidified to pH 1.8 with HCl and scanned with a double-beam spectrophotometer (Hitachi U-2000). Absorbance at 310 nm was used to estimate total catecholates and absorbance at 380 nm was used to estimate azotobactin (Page & Huyer, 1984). Total cell protein in the pellet was measured by the method of Lowry *et al.*, (1951) or Bradford (1976). Siderophore production by different strains was compared using A_{310} and A_{380} per mg cell protein.

2.4 Purification of azotobactin from *A. vinelandii*

Strains UW and UA22 were grown in OFeBBGN with 1 µM ferric citrate added to promote Fe-limited growth. After 40 h incubation the culture was centrifuged (10,400 xg in a RC-5 Sorval centrifuge using a GSA rotor). The supernatant, containing the siderophores, was collected and acidified with 6 N HCl to obtain a pH of 1.8-1.9. The acidified supernatant was then transferred to a separatory funnel and an equal volume of ethyl acetate was added. The separatory funnel was shaken vigorously ten to fifteen times and the two phases were allowed to separate. The bottom (aqueous) and the top (ethyl acetate) layers were collected and the extraction of the aqueous phase was repeated. The aqueous layers were pooled and filtered through an 0.2 µm pore filter. The filtrate was neutralized to pH 7.0 with 6 N NaOH, and loaded onto a DEAE Sephacel (Pharmacia) column. The remaining sample not loaded onto the column immediately was kept frozen at - 20°C.

DEAE Sephacel beads were washed and soaked overnight in packing solution (2 M NaCl + 0.5 mM EDTA). For packing the column, the outlet tubing was initially kept closed. About 50% of the slurry of beads was poured into the column with a help of a glass rod, so as not to introduce air bubbles. When the column was full, the outlet was opened and the beads were allowed to settle. Flow rate was maintained around 100 ml/h. After the column was packed, it was washed with two column volumes of 2 M

NaCl and then with Milli-Q water overnight. This was followed by a series of washes with two column volumes each of 0.1 N HCl followed by Milli-Q water, then with 2 M NaCl and then with Milli-Q water again. The last wash (with Milli-Q water) was done until the eluant had a pH of 3.5.

The column was loaded with the sample at a very slow flow rate (50 ml/h). The column was then washed with Milli-Q water followed by 0.004 N HCl. The sample was eluted with 0.04 N HCl. The fractions were collected in 16 mm tubes (approx. 8 ml per tube). Those with absorbance readings at 380 nm of more than 0.1 were pooled together and freeze-dried (Virtis Co, USA). The sample containing azotobactin was protected from light during the entire procedure.

The column was regenerated for a second run by washing it with Milli-Q water overnight, followed by 2 M NaCl + 0.5 mM EDTA, Milli-Q water, 0.1 N HCl, Milli-Q water, 2 M NaCl + 0.5 mM EDTA, and finally with Milli-Q water.

2.5 Isolation of chromosomal DNA from *A. vinelandii*

Genomic DNA of *A. vinelandii* was isolated as described by Robson *et al.* (1984). Three hundred ml of a 19 h culture grown in BBGN was centrifuged at 10,400 xg for 10 min. The cell pellet was washed once with PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0) and resuspended in 12 ml TES (10 mM Tris, 50 mM EDTA, pH 8.0) and sodium dodecyl sulfate (SDS) added to make up a final concentration of 1.5% (w/v)). The suspension was incubated at 30 °C for 10 min after which Pronase E (Sigma, Type XXV) (1 mg/ml) was added. The solution was incubated at 37 °C for 2-3 h. The DNA was precipitated with 2 volumes of ACE (sodium acetate, 0.2 M, in 90% v/v ethanol) and collected by spooling onto a glass rod. Extra ethanol was drained from the spool and the DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) overnight. The solution was extracted three times with STE-saturated phenol. [Phenol was saturated with STE (100 mM NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) so that the DNA would remain in the aqueous phase. STE-saturated phenol was prepared by mixing equal volumes of phenol and STE in a separatory funnel and allowing the mixture to settle. The aqueous phase was discarded. The extraction was repeated at least three or four times until the aqueous phase had a pH of 8.0.] After phenol extraction, the aqueous phase containing DNA was extracted twice with chloroform:isoamyl alcohol (24:1 ratio of chloroform to isoamyl alcohol). The DNA

was reprecipitated with 2 volumes of ACE, collected on a glass rod and then dissolved in 5 ml of TE buffer. RNA in the sample was removed by adding DNase-free pancreatic RNase (20 µg/ml) and incubating for one hour at 37 °C. To obtain DNase-free RNase, the procedure described by Sambrook *et al.* (1989) was used. Pancreatic RNase (RNase A) was dissolved at a concentration of 10 mg/ml in 10 mM Tris-HCl, pH 7.5. The solution was heated to 100 °C for 15 min, allowed to cool slowly to room temperature and then dispensed in aliquots which were stored at -20 °C.

To estimate the concentration of DNA, the absorbance was determined at 260 nm. (A_{260} of a 50 µg/ml solution of pure double stranded DNA is 1.0 (Sambrook *et al.*, 1989)).

2.6 Plasmid isolation and analysis

Plasmid DNA was purified from the *E. coli* strains using a modified Birnboim and Doly (1979) small scale alkaline lysis technique as described by Sambrook *et al.* (1989). The organisms were grown overnight in liquid medium. The culture was transferred to a 1.5 ml Eppendorf tube and centrifuged for 2 min in a microcentrifuge at 13,000 rpm. The supernatant was aspirated using a vacuum line and discarded. For low copy number plasmids, another 1.5 ml culture was added to the same tube and the cells were pelleted. The pellet was resuspended in 100 µl of Solution #1 (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0; sterile). The suspension was mixed well by vortexing. Then 200 µl of freshly prepared Solution #2 (0.2 M NaOH, 1% w/v SDS) was added and the contents were mixed gently by inverting the tubes. After 5 min incubation on ice, 150 µl of ice-cold Solution #3 (60 ml of 5 M potassium acetate solution containing 11.5 ml of glacial acetic acid and 28.5 ml water, final pH 4.8) was added and the contents were mixed by inverting the tubes several times. After 5 min incubation on ice, the tubes were centrifuged for 5 min at 4 °C. The supernatant was transferred to a clean Eppendorf tube and an equal volume of phenol:chloroform (1:1) mixture was added. The contents were mixed by vortexing and the tubes were centrifuged for 3 min at room temperature. [The phenol:chloroform mixture was made with TE-saturated phenol and a mixture of choloroform and isoamyl alcohol, in the same way as STE-saturated phenol, except that TE was used as the saturating buffer]. After the extraction, the supernatant was collected in a fresh tube and DNA was extracted once with chloroform:isoamyl alcohol (24:1) in a similar way. Finally, the DNA was precipitated by adding 1/10 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol at room

temperature for 5 min. DNA was collected by centrifuging the tube in a microcentrifuge at 13,000 rpm for 15 min at 4 °C. The resulting pellet was washed with cold 70% ethanol to remove salts and air dried. The pellet was redissolved in TE buffer containing DNase-free RNase (to a final concentration of 20 µg/ml).

For large scale plasmid preparations, the starting culture volume was 500 ml. The cells were harvested, and then resuspended in 25 ml of ice cold Solution #1 (with 4 mg /ml lysozyme). The rest of the isolation procedure was the same as that described for small scale preparation, except that the volume of reagents was adjusted accordingly.

For obtaining ultrapure plasmid DNA, a cesium chloride-ethidium bromide procedure was carried out as described in Sambrook *et al.* (1989). Cesium chloride (8 g) was dissolved in 8 ml of DNA sample in TE buffer and then 0.8 ml of ethidium bromide (10 mg/ml) was added. The solution was centrifuged at 7,800 xg for 5 min at room temperature using a Sorval SS34 rotor. The supernatant was transferred to an ultracentrifuge tube (Beckman quick seal tube for type 50 Ti rotor). The top of the supernatant was layered with paraffin oil to exclude air bubbles before the tube was sealed with the sealer (Beckman tube sealer). The tube was centrifuged in an ultracentrifuge at 36,000 rpm for 48 h at 20 °C using the 50 Ti rotor. Two bands of DNA were located in the center of the gradient under UV light. The upper band consisted of chromosomal DNA and nicked circular plasmid DNA while the lower band consisted of closed circular plasmid DNA. Both the DNA bands were collected separately using a 21 gauge hypodermic needle. To remove ethidium bromide, the plasmid DNA layer was extracted with water-saturated butanol until colorless. This was followed by dilution with 2 volumes of water, and 6 volumes of 95% ethanol. After incubating on ice for 15 min, the DNA was pelleted by centrifugation, using the Sorval SS34 rotor, at 12,000 xg for 15 min at 4 °C. The DNA pellet thus obtained was washed with cold 70% ethanol, redissolved in TE buffer and stored at -20 °C.

Table 2.2 shows a list of vectors and plasmid constructs used in the study.

2.7 Electrophoresis of DNA

The DNA was analyzed by running a small aliquot on an agarose gel as described by Sambrook *et al.* (1989), using the TAE buffer system (per liter: 4.84 g Tris base, 1.142 ml glacial acetic acid, 2 ml 0.5 M EDTA pH 8.0, water to 1 liter). The percentage of

Plasmid	*Parent Plasmid*	*Host*	*Selective Markers*	*Source*	*Identity / Uses / Insert*	*Reference*
M13 mp 18	-	DH5α F'	Amp	B. Leskiw	Phage vector for single stranded sequencing	Messing, 1983
M13 mp19	-	DH5α F'	Amp	B. Leskiw	Phage vector for single stranded sequencing	Messing, 1983
pCON6	-	DH5 α	Amp	J.B. Neilands	*iuc*A containing fragment, for gel retardation studies	de Lorenzo *et al.*, 1988a
pIJ2925	-	DH5 α	Amp	B. Leskiw	Cloning vector	Janssen & Bibb, 1993
pIJMPCR2	pIJ2925	DH5 α	Amp	This sudy	255 bp *Hin*dIII-*Eco*RI fragment of PCR2	This sudy
pKSS	-	DH5 α	Amp	P. Kast	Cloning vector, *E. coli pheS* gene	Kast, 1994
pM110	pIJ2925	DH5 α	Amp	This study	635 bp *Hin*dIII-*Sal*I fragment of UA22 DNA	This study
pM112	pQF50	JM106	Amp	This study	647 bp *Bgl*II-*Sal*I fragment of pM110, β-Gal	This sudy
pM114	pQF50	JM106	Amp	This sudy	320 bp *Sma*I-*Hin*dIII fragment of UA22 DNA	This sudy
pMKSS	pQF50	JM106	Amp	This study	1.2 kb *Bam*HI-*Sal*I fragment of *E. coli pheS* gene	This sudy
pMPCR2	pQF50	JM106	Amp	This sudy	270 bp *Hin*dIII-*Bgl*II fragment of pIJMPCR2	This study
pMJH3	-	DH5 α	Amp	P. Bishop	*anfH* of *A. vinelandii*, for gel retardation studies	Joerger *et al.*, 1989
pQF50	-	JM106	Amp	M. Farinha	Promoter probe vector	Farinha & Kropinski, 1990
pMVW94	pUC119	MV1193	Amp , Kan, Lux$^+$	M. Vande Woestyne	12.9 kb UA22 DNA, Lux$^+$	Vande Woestyne & Page, Unpublished
pMVW98	pUC119	MV1193	Amp	M. Vande Woestyne	1.3 kb UA22 DNA	Vande Woestyne & Page, Unpublished
pUC119	-	DH5 α	Amp	Lab stocks	Cloning vector, *lac* promoter for Gel retardation	Vieira & Messing,

Table 2-2 List of plasmids and plasmid constructs used in this study.

agarose (Low EEO, Boehringer Mannheim) used was 0.8%, unless otherwise stated. *Hin*dIII and *Pst*I fragments of λ phage were used as molecular weight standards.

For acrylamide gels, 5% polyacrylamide gel was used with a TBE buffer system (0.1 M Tris-HCl, pH 8.0; 1 mM EDTA; and 60 mM boric acid). After electrophoresis, the gels were stained with ethidium bromide (10 µg/ml) and destained with distilled water. Photographs were taken using UV illumination and a red-orange filter or using the Photoimager (The Imager, Appligene-Oncor).

2.8 Elution of DNA fragments from gels

From agarose gels:

(a) PEG-ethidium bromide method (Zhen & Swank, 1993): This procedure involves monitoring the migration of DNA through normal agarose gels containing ethidium bromide using long-wave UV light. The DNA fragments were separated by electrophoresis in an agarose gel (0.8% - 1%; electrophoresis grade) that contained 0.5 µg/ml ethidium bromide. The DNA band of interest was visualized by illumination with long-wave UV light. Using a sharp scalpel blade, a rectangular trough was cut to the bottom of the gel tray directly in front of the leading edge of the band of interest. The gel slice and any small bits of agarose were removed. The trough was then filled with about 300-450 µl of 15% PEG/TAE [15 g of polyethylene glycol 8000, was added to 50 ml of 2x TAE buffer, water to 100 ml and autoclaved. After cooling, ethidium bromide was added to obtain a final concentration of 0.5 µg/ml]. Electrophoresis was continued with a voltage of about 20-25 V/cm. The mobility of the DNA fragment of interest was checked periodically using a long-wave UV lamp. When the DNA band of interest moved into the center of the trough, electrophoresis was stopped and the DNA-containing PEG/TAE solution was pipetted into a microcentrifuge tube. This was followed by two phenol:chloroform extractions. The DNA was then precipitated with 1/10 volume of sodium acetate and 2.5 volumes of 95% ethanol.

(b) Glass wool column method: Once the band of interest was located by ethidium bromide staining after agarose gel electrophoresis, the band was cut out with a sharp scalpel and transferred to a small 0.5 ml Eppendorf. Prior to this, a tiny hole was made at the bottom of the 0.5 ml tube with a 23.5 gauge needle. The tube was packed half full with silanised glass wool and placed inside a lidless 1.5 ml Eppendorf tube. The sample was centrifuged at 13,500 rpm in a microcentrifuge for 15 min at 4 ^{0}C. The liquid

(containing the DNA) collected in the 1.5 ml tube was extracted with phenol:chloroform and precipitated.

From polyacrylamide gels ("crush and soak" method):

After running and staining the polyacrylamide gel, the band of interest was located using long-wave UV light and cut out using a sharp razor blade. The gel slice was placed on a glass plate, chopped into very fine pieces, and transferred to a 1.5 ml Eppendorf tube. One volume of elution buffer (0.5 M ammonium acetate containing 1 mM EDTA, pH 8.0) was added and the tube was incubated at 37 °C in a tube roller (the Eppendorf tube was placed in a 16 mm test tube) overnight. The sample was centrifuged in a microcentrifuge at 13,000 rpm for 10 min at 4 °C and the supernatant was transferred to a fresh tube without disturbing the acrylamide fragments. The acrylamide pellet was resuspended in 0.5 volume of elution buffer and the tube was recentrifuged. The two supernatants were combined and the DNA was precipitated with 2.5 volumes of ethanol, redissolved in 200 µl of TE and 20 µl of 3 M sodium acetate, and precipitated again. The pellet was rinsed with 70% ethanol and dried before resuspending in a small volume of TE.

2.9 Quantitation of DNA

2.9.1 Quantitation of DNA in terms of ng or µg.

To quantify the total DNA present in a given sample, the following procedures were used:

(1) "Eyeballing": The DNA sample was run on an agarose gel along with the known amounts of the marker DNA (for example λ-*Hin*dIII or λ-*Pst*I). After staining the gel, the intensity of the band of interest was compared with the band in the marker lane of closest size. This gave a rough estimate of the amount of DNA present in the given DNA sample. This procedure was used for estimating the concentrations of the vector and the insert DNA to be used for ligation reactions.

(2) A_{260}: For quantifying DNA samples like synthetic oligonucleotide primers, this procedure was followed. The DNA sample was adequately diluted. The absorbance of the sample at 260 nm was recorded using the spectrophotometer. For oligonucleotides an A_{260} of 1 represented 30-35 µg . For other DNA, A_{260} of 1 represented 50 µg.

(3) Ethidium bromide method: A standard curve was made using known concentrations of calf-thymus DNA, and the concentration of the given sample was calculated by comparison. The standard DNA at concentrations 0, 10, 20, 30, 50, 75, 100, 150, 250 and 500 ng was made to 950 µl with 5 mM Tris-HCl (pH 8.1) containing 0.5 mM EDTA. Next, 50 µl of ethidium bromide (10 µg /ml) solution was added and the tubes were incubated at room temperature in the dark for 5-10 min. The increase in emission at 600 nm was read with a Hitachi F2000 fluorescence spectrophotometer by setting the excitation wavelength at 525 nm. A standard curve was made by plotting the emission at 600 nm versus the amount of DNA in ng/ml in each tube. The concentration of DNA in the sample of interest was calculated from the graph.

2.9.2 Quantitation of DNA in picomoles or Molar concentrations

(A) For synthetic oligonucleotides. The extinction coefficient was calculated according to the following formula:

$$E_o = A^n \times 15.4, C^n \times 9.2, G^n \times 11.4, T^n \times 9.7$$

where n is the number of that particular base present in the oligonucleotide. After adding the values, E_o is obtained. The value of A_{260}/ E_o gives the value of DNA concentration in mM units.

(B) To calculate the concentration of a double-stranded DNA fragment of X base pairs in moles, the size of the fragment (in bp) was multiplied with 660 Daltons (molecular weight of one bp). The value was obtained in terms of Daltons (which is equivalent to g/mole) and converted to ng/pmoles. The concentration of the given DNA sample in terms of ng was substituted and picomole equivalents calculated.

2.10 Transformation of *E. coli*

Competent cells of *E. coli* strains were prepared according to the procedure described by Chung *et al.* (1989) (TSS method) or by $CaCl_2$ method (Sambrook *et al.*, 1989).

For the TSS method, an overnight culture was used as an inoculum. The culture was diluted 1:100 in LB medium and the cells were grown to an optical density (OD_{600}) of 0.3 - 0.4. The culture (1.5 ml) was centrifuged in an Eppendorf tube at 6,500 rpm for 3 min at 4 ^{0}C and the supernatant was discarded. The pellet was redissolved gently in

100 µl of LB and 100 µl of ice cold 2x TSS broth (LB containing 20% PEG 8000, 10% dimethyl sulfoxide and 100 mM $MgCl_2$) were added. After the cells were incubated on ice for 30 min, they were ready for transformation. DNA (1-5 µl) was added to the competent cells and the mixture was allowed to sit on ice for 25 min. Next, 900 µl of LB was added to the mixture and incubated at 37 °C for one hour. After the transformed cells had recovered, the cells (100 - 200 µl) were plated on to selective media of LB + antibiotic and the plates were incubated overnight at 37 °C.

For the $CaCl_2$ method, an overnight culture was diluted 1:100 with LB medium and grown (20 ml/50 ml flask) to an OD_{600} of 0.3 - 0.4. The cells were chilled on ice for 15 min, harvested by centrifugation in a microcentrifuge at 2,000 rpm for 10 min at 4 °C, washed twice with 5 ml of cold 0.1 M $CaCl_2$, and pelleted by centrifugation as before. The cells were then resuspended gently in 1 ml of $CaCl_2$ and stored at 4 °C for 12-24 h for maximal competence. Later, 100 µl of cells was mixed with 2-10 µl of DNA. The mixture was incubated on ice for 25 min and heated at 42 °C for 1 min to facilitate DNA uptake. Then, 0.9 ml LB was added to the mixture and incubated at 37 °C for 1 h. The transformed cells were plated on LB plates with antibiotics. Antibiotic-resistant colonies were picked after overnight incubation at 37 °C. Small scale plasmid preparations, as described above, were carried out on a few colonies to confirm the presence of plasmid.

2.11 DNA sequencing

The 1.3-kb *Pst*I-*Sma*I fragment of *Azotobacter* DNA from pMVW98 was used for sequence determination. This fragment was sequenced in three parts, taking advantage of the internal *Sal*I and *Hind*III sites. DNA fragments were subcloned in the M13 phage vectors mp18 and mp19 to isolate single-stranded DNA.

2.11.1 Subcloning in M 13 phage

One microgram of M13 mp18 and M13 mp19 (Boehringer) vector DNA was digested with two restriction enzymes, *Hin*dIII and *Sal*I. Similarly, pMVW98 was digested with the same enzymes. Since the buffer systems for these enzymes were different, the sample was digested with *Hin*dIII first. After phenol:chloroform extraction and reprecipitation, the DNA was digested with *Sal*I. T4 DNA ligase (Boehringer) was used for ligation and a 2:1 ratio of insert:vector in a 10 µl final volume. Buffer and enzyme in the ligation reaction was used according to the manufacturer's

recommendations. The final DNA concentration in the ligation mixture was between 20 and 60 ng/µl.

Similarly, the *Sma*I-*Hin*dIII fragment of pMVW98 was subcloned into mp18 and mp19 phage vectors.

2.11.2 Transfection

Competent cells of *E. coli* strain DH5αF' were made according to the $CaCl_2$ method. Soft agar (8 g Bacto agar, 10 g Bacto tryptone, 8 g NaCl, water to 1 liter) was made and dispensed (3 ml) in 13 × 100 mm tubes. The ligation mixture was added to 200 µl of competent cells, mixed well and incubated on ice for 30 min. The melted soft agar was placed in a dry thermal incubator set at 45 °C. The competent cells and ligation mixture was heated at 42 °C for 1 min. During heat shocking, 50 µl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 10 µl of IPTG (isopropyl β-D-thiogalactopyranoside, Sigma biochemicals) was added to the soft agar. After heat shocking, 200 µl of plating cells (overnight grown DH5α F') and 180 µl (90%) of the transformed cells were added to one soft agar tube and 200 µl of plating cells and 20 µl (10%) of the transformed cells added to the other. These two soft agar mixtures were poured quickly onto two hard agar plates (15 g bacto agar, 10 g bacto tryptone, 8 g NaCl, water to 1 liter). After the soft agar had solidified, the plates were incubated at 37 °C overnight. The appearance of white plaques indicated transformants with the insert while blue plaques showed the presence of phage without the insert.

2.11.3 Preparation of single-stranded DNA

To obtain single-stranded DNA for sequencing, the positive clones of mp19 and mp18 were used. An *E. coli* strain containing the F' factor (DH5α F' or MV1193 F') was grown overnight in 2x YT broth (1.6% w/v tryptone, 1% w/v yeast extract, 0.5% w/v NaCl) or LB (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) and diluted 1:100 in 2 ml of the same medium (in 16 × 150 mm tubes). This was inoculated with the appropriate plaque (using a toothpick) and allowed to grow for 4.5 to 5 h at 37 °C on a tube roller. The culture (1.5 ml) was transferred to an Eppendorf tube and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to another tube containing 250 µl PEG/ammonium acetate (20% PEG 8000 and 3.5 M ammonium acetate). This tube was mixed well and incubated on ice for 30 min before centrifuging for 15 min at 4 °C.

The pellet thus obtained was resuspended in 100 µl of TE and left at room temperature for 5 min. This was followed by extractions with neutral phenol and diethyl ether. After removing the ether layer by aspiration, the phage DNA in the aqueous layer was precipitated by adding 40 µl of 7.5 M sodium acetate and 220 µl of 95% ethanol. After thorough mixing, the tube was incubated in a dry ice ethanol bath for 1 h or at -20 °C overnight. The DNA was pelleted and washed with 70% ethanol. Finally, the DNA pellet was dissolved in 50 µl of sterile Milli-Q water.

2.11.5 Sequencing of UA22 DNA

The single-stranded DNA template was sequenced according to the chain termination method of Sanger *et al.* (1977) modified by Tabor and Richardson (1987) for use with Sequenase (version 2.0 kit, United States Biochemicals, Cleveland, Ohio, USA). Oligonucleotide primers were synthesized as needed[1] , to allow sequencing along both strands of the cloned DNA. An additional 228 bp sequence upstream of the *Sma*I site was attained by double stranded automated DNA sequencing using[1] pMVW94 as the template.

For the sequencing reaction:

(a) Annealing mix: 4 µl of template DNA was mixed with 1.5 µl of water, 2 µl of Sequenase reaction buffer, and finally 2.5 µl of the primer. This annealing mix was heated for 2 min at 65 °C, allowed to cool slowly to below 35 °C over 30 min and chilled on ice.

(b) While cooling, 4 tubes were labeled and 2.5 µl of each of the termination mixes was added (ddGTP, ddATP, ddTTP, and ddCTP).

(c) The 4 termination tubes were prewarmed at 37 °C.

(d) Labeling reaction: To the ice-cooled 10 µl annealing mix, 1 µl of Mn buffer, 1 µl DTT, 2 µl of the deaza (7-deazadeoxyguanosine-5'-triphosphate) mix, 0.5 µl ^{35}S dATP and 1:8 diluted Sequenase enzyme was added. The reaction was started by adding the enzyme and incubating for 2 min at room temperature.

(e) Termination reaction: After 2 min, 3.5 µl of the labeling reaction was added to each termination tube, mixed well and the reaction was allowed to continue for another 2 min at 37 °C.

(f) The reaction was stopped by adding 4 µl of the stop solution and storing on ice.

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[1]Molecular Biology Services, Department of Biological Sciences, University of Alberta

(g) The samples were heated to more than 80 °C for 5-10 min immediately before loading onto the sequencing gel (2-4 µl per lane).

For 6% gel working solution: Urea 460 g (ultra pure, ICN Biomedicals Inc.) in 100 ml of 10x TBE (109 g Tris base, 55 g boric acid, 9.3 g EDTA, volume made up to 1 liter with Milli-Q water; final pH 8.3), 150 ml of 40 % acrylamide/bis gel mix (19:1, BioRad Laboratories). The volume was made up to 1 liter with Milli-Q water.

For the plug: 10 ml 6% gel working solution, 50 µl TEMED (N,N,N',N'-tetramethylethylenediamine) and 50 µl freshly prepared 25 % ammonium persulfate (APS).

For the running gel: 50 ml of 6 % gel working solution, 75 µl TEMED and 75 µl of APS. The gel was run in 1x TBE buffer system at constant power of 40 W (3,000 V, 200 milliamps, Bio-Rad powerpack).

After electrophoresis, sequencing gels were placed in a fixing solution (10% v/v methanol, 10% v/v acetic acid) for 10 to 15 min. The gel was lifted onto Whatmann (3MM) filter paper and dried using a Biorad Model 583 gel drier. Radioactive bands were visualized by exposing the dried gel to a Kodak X-OMAT AR film at room temperature overnight. The film was developed using a FUJI RGII X-ray film processor.

2.12 Gene bank searches

The computer software used for the analysis of DNA and protein sequences was DNA Strider, designed and written by C. Marck (Commissariat a l'Energie Atomique, France). Protein and DNA similarities were determined using the BLAST (Altschul *et al.*, 1990 and Gish & States, 1993) programs (BLASTX & BLASTN) which were obtained from the INTERNET.

2.13 Subcloning in the promoter probe vector pQF50

The 635 bp *Sal*I-*Hin*dIII fragment of pMVW98 was subcloned into the promoter probe vector pQF50. There were no suitable restriction enzyme sites in the multiple cloning site of pQF50 for the insertion of this fragment to have the iron-regulated promoter in the correct orientation with *lacZ*. Therefore, the 635 bp *Sal*I-*Hin*dIII fragment was first inserted into the same sites in vector pIJ2925. The resulting clone

pM110 (in *E. coli* DH5α) was identified using blue/white selection and confirmed to contain the correct sized fragment by restriction enzyme digestion. Next, pM110 was digested with *Bgl*II and *Sal*I enzymes to excise a 647 bp fragment. The presence of the 635 bp *Sal*I-*Hind*III fragment within this larger fragment was reconfirmed by restriction enzyme digestion. The 647 bp *Bgl*II-*Sal*I fragment was subcloned into pQF50. The resulting *E. coli* JM106 clone (pM112) formed blue colonies on LB plates containing X-gal.

To check whether the *E. coli pheS* gene also had an internal promoter, vector pKSS which had a promoterless *pheS* gene positioned downstream of the *lac* promoter, was used. This promoterless *E. coli pheS* gene was excised as a 1.2 kb *Bam*HI-*Sal*I fragment and ligated into the *Bam*HI-*Sal*I sites of pQF50. The resulting clone pMKSS formed blue colonies on LB plates containing X-gal.

In order to test for promoter activity in the 320 bp *Sma*I-*Hin*dIII fragment of pMVW98, this fragment was cloned into *Sma*I-*Hin*dIII sites of pQF50. This construct formed white colonies when plated on LB medium containing X-gal.

2.14 Generation of PCR fragments

A 286 bp PCR1 fragment of UA22 DNA was amplified using two primers: WJP8 and WJP9. WJP8 had a sequence 5'- GGTGCGCACCTGAACCG –3' and WJP9 was a 27-mer with a 10 base non-homologous extension at the 5' end (sequence in lower case) 5'- gcgcgaattcAAGTTGGCCGCCGAGCG –3'. The latter had an *Eco*RI site (sequence underlined) engineered in the non-homologous extension.

Five PCR reactions were set up in 0.5 ml Eppendorf tubes as follows:

2 µl each of the two primers (10 picomoles /µl)
10 µl of 10x PCR buffer
16 µl of 1.25 mM dNTPs
1 µl of 100 mM $MgCl_2$
10 µl of 1 ng/µl concentration of the template DNA (pMVW98)
DMSO (different volumes in each tube 0, 2, 4, 6, and 8 µl)
Milli-Q water to 99.5 µl and
0.5 µl Taq DNA Polymerase (1-5x10^3 units/ml, Boehringer Mannheim)

After adding the enzyme, three drops of sterile mineral oil were layered on the top. The PCR machine (PHC-2, Techne) was programmed as follows: initial heating at 95 °C

for 5 min and then 30 cycles of: 30 seconds at 95 °C, 30 seconds at 52 °C and 1 min at 72 °C. At the end of the 30 cycles (2.5 to 3 h), the tubes were stored at 4 °C.

To remove the top oil layer, the tube was kept in -70 °C for 15 min. Since the aqueous layer was frozen (while the oil layer was not), the oil layer was quickly aspirated off using vacuum. The DNA sample was extracted with chloroform twice to remove any remaining oil. A small aliquot was electrophoresed on an agarose gel to check for the correct sized fragment (265 bp).

To purify the correct sized fragment from the other smaller fragments, the samples were loaded on a 5 % polyacrylamide gel. After running and staining the gel, the band of interest was purified using the "crush and soak" method (section 2.8).

Another PCR fragment (265 bp; PCR2) was amplified using WJP9 and WJP10 as the primers; the rest of the procedure was identical to the one described above. WJP10, a 27-mer, had the sequence 5'- cgcgg<u>aagct</u>TGCAGCAGCATATTGGC –3', containing 10 bases of non-homologous extension at the 5' end (sequence in lower case), with a *Hin*dIII site engineered in it (sequence underlined).

2.15 Subcloning of PCR2 in pQF50

Oligonucleotide primers WJP10 and WJP9 (as shown above) had a *Hin*dIII and an *Eco*RI site engineered into them, respectively. To obtain the correct orientation of the iron-regulated promoter (IRP) with respect to the promoterless *lacZ* in pQF50, the PCR fragment (PCR2) was first cloned into pIJ2925 and subcloned in pQF50.

PCR2 and the vector pIJ2925 were each digested with *Hin*dIII and *Eco*RI enzymes. A ligation reaction was setup, with 3x more insert than vector at 15 °C for 12-16 h. DH5α competent cells were transformed with the ligation mix and plated on LB plates containing Amp, X-gal and IPTG. White colored colonies were selected. A few probable positive clones were picked and grown overnight in LB containing Amp. Small scale plasmid isolation was performed to confirm the presence of a recombinant plasmid. The positive clone was named pIJMPCR2.

pIJMPCR2 was isolated and digested with *Hin*dIII and *Bgl*II. The 270 bp fragment was purified from the gel using the trough method (PEG-ethidium bromide; section 2.8). Next, pQF50 was converted to the linear form by digesting with *Hin*dIII and *Bgl*II. The PCR2 fragment was ligated into pQF50 and the ligation mixture was transformed into

JM106 competent cells. The transformants were selected on LB Amp plates coated with X-gal beforehand to check for any promoter activity within the insert. A number of white colonies and a few blue colonies were obtained. A few of each kind were grown overnight in Amp supplemented LB. Plasmid DNA was isolated and digested with *Hin*dIII and *Bgl*II. The results showed that the blue colonies contained the correctly sized insert (PCR2) whereas the white ones had only the recircularized vector. One blue, positive clone was called pMPCR2.

2.16 β-Galactosidase assays

E. coli cultures supplemented with appropriate antibiotics were incubated overnight in LB medium. Each culture was diluted (1:100) into two types of media (50 ml/ 225 ml flask): one that contained 250 μM of deferrated EDDHA per ml (low-iron culture) and another with 50 μM ferric citrate (high-iron culture). The cultures were incubated at 37 °C and samples were withdrawn after 2 h and every hour thereafter. Quantitative assays to determine β-galactosidase activity were performed as described below (Miller, 1972).

After 2 h, three ml of culture were withdrawn, dispensed into a test tube (13 × 100 mm) and kept on ice for 20 min to prevent further growth of the cultures. After the 20 min on ice, 1 ml of the culture was used to determine the optical density at λ 600 nm and 1 ml was used in the assay. Culture (0.5 ml) was added to 0.5 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercapto ethanol; pH adjusted to 7.0). Next, 2 drops of chloroform and 1 drop of 0.1% SDS was added and the tube containing the mixture was vigorously shaken using a vortex mixer for 30 sec followed by incubation at 28 °C for 5 min. The reaction was started by adding 200 μl of the substrate ONPG (4 mg o-nitrophenyl β-D-glucopyranoside per ml of 0.1 M phosphate buffer pH 7.0) and terminated with 0.5 ml 1 M sodium carbonate (to increase the pH) after sufficient yellow color had developed. The duration of the reaction was noted. Absorbance (at 420 nm and 550 nm) was recorded for each tube and enzyme activity in terms of Miller units was calculated according to the following formula:

$$\frac{1000 \times (A_{420} - 1.75 \times A_{550})}{t \times v \times A_{600}} = \text{β-galactosidase activity in Miller units}$$

where the correction factor for light scattering for *E. coli* is 1.75, t is the duration of reaction in min, and v is the volume of culture (in ml) used in the assay.

2.17 Gel mobility retardation assay

Binding of the *E. coli* Fur protein (kindly provided by J.B. Neilands, Department of Biochemistry, University of Berkeley) to the IRP of UA22 was assayed by a polyacrylamide gel electrophoresis retardation method (de Lorenzo *et al.*, 1988).

A non-radioactive procedure was followed in which the 647 bp *Pst*I-*Sal*I fragment from pM110 (purified from an agarose gel) was used as the target DNA. Other target DNA fragments included a 250 bp *Eco*RI -*Pvu*II fragment of pCON6, containing the aerobactin (*iucA*) operator sequence, as a positive control. A 322 bp *Pvu*II fragment of pUC119 (containing the *lac* promoter) and a 670 bp *EcoR*I-*Xho*I fragment of pMJH3 (containing the nitrogen-regulated promoter of the *A. vinelandii* nitrogenase gene *anfH*) were used as negative controls. *Pst*I-*Sal*I digested pKSS, containing part of the promoterless *pheS* gene from *E.* coli, was also used.

The 15 µl assay mixture consisted of 10 µl binding buffer, 1-2 µl (containing approximately 0.1 nM) target DNA and 2-3 µl (containing various amounts) of the Fur protein. The binding buffer contained 10 mM bis-(2-hydroxyethyl)imino-tris (hydroxymethyl)methane (Bistris)/boric acid pH 7.5, 5 µg/ml salmon sperm DNA (sonicated and denatured, Sigma Chemical Co.), 5% glycerol, 100 µg/ml bovine serum albumin, 1 mM $MgCl_2$, 100 µM $MnCl_2$ and 40 mM KCl. After 15 min incubation at 37 °C, the assay mixture was loaded (without tracking dye) on a 5% polyacrylamide gel in 20 mM Bistris/boric acid pH 7.5, containing 100 µM $MnCl_2$. Electrophoresis (16 cm x 17 cm) was performed at 200 Volts for 3-4 h. DNA bands were visualized by staining with SYBR™-Green nucleic acid gel stain (Molecular Probes Inc., Eugene, Oregon) and photographed under UV light.

In a second method, the following ^{32}P end-labeled DNA fragments were used: 255 bp *Hin*dIII-*Eco*RI pMPCR2 fragment, 670 bp *Eco*RI-*Xho*I fragment of pMJH3 (containing the nitrogen-regulated promoter of the *A. vinelandii* nitrogenase gene *anfH*) and 250 bp *Eco*RI-*Pvu*II fragment of pCON6 (containing the aerobactin (*iucA*) operator sequence, as a positive control). The fragments were purified from polyacrylamide gels by the "crush and soak" method. The DNA present in the samples was quantified according to the ethidium bromide method and dephosphorylated with alkaline phosphatase before end-labeling with polynucleotide kinase. The procedure for gel retardation was essentially the same as described for the non-radioactive assay. Less than 0.1 nM of the end-labeled target DNA was used with varying concentration of the Fur protein. As a negative control, the same experiment was carried out as before, but

Mn^{+2} was excluded from the reaction system. After the gel was electrophoresed, it was dried in a gel drier (Biorad model 583) and exposed to X-ray film for 5-20 h (for autoradiography) or exposed to the phosphor screen for 1-2 h and scanned using a phosphorimager (Phosphor Imager 445 S1, Molecular Dynamics).

The phosphorimager files (in tiff format) were transferred into Adobe Photoshop LE (Adobe Systems Inc., 1995 Power Mac version) to optimize the contrast and background.

2.18 Southern hybridization

Transfer of DNA from agarose gels to nylon membranes

Each gel was electrophored overnight at 30 V, stained with ethidium bromide and photographed. The procedure as described by Southern (1975) was followed for transferring separated DNA fragments from an agarose gel to a nylon membrane. The gel was soaked in 0.25 M HCl for 15-20 min to depurinate the DNA, and rinsed with Milli-Q water. Next, the DNA fragments were denatured by soaking the gel twice in denaturation solution (1.5 M NaCl in 0.5 M NaOH) for 15 to 20 min, at room temperature. The gel was rinsed with water and placed in neutralization buffer (1.5 M NaCl, 0.5 M EDTA in 1 M Tris-HCl, pH 7.2) at room temperature with gentle shaking (replaced twice in 20 min). DNA from the gel was transferred to a nylon membrane by the capillary blotting method (Sambrook *et al.*, 1989). For blotting, 20x SSC (3 M NaCl in 0.3 M tri-sodium citrate) was used. After overnight transfer, the nylon membrane was allowed to air dry before baking in an 80 °C vacuum oven for 2-3 h.

Radioactive labeling of the DNA probe (random primer labeling)

The procedure as recommemded by the Boehringer labeling kit instructions was used. DNA (25-50 ng) in 9 µl Milli-Q water was denatured by heating up to 90 °C for 10 min followed by cooling rapidly on ice. To the cooled and denatured DNA was added 2 µl of vial #5 of Boehringer labeling kit (hexanucleotide mix), 3 µl of dNTP mix (dATP, dTTP, and dGTP) and 5 µl of α ^{32}PdCTP (10 µCi/µl). The contents of the tubes were mixed and 1 µl of Klenow fragment of DNA polymerase was added. The reaction was allowed to take place at room temperature overnight or at 37 °C for 4 h. The unincorporated nucleotides were removed by passing the sample through a G-50 sephadex column prepared in TE buffer (pH 8.0). The column of about 3.5 ml was packed in a 5 ml Eppendorf tip with a glass bead at the bottom of the tip. Before loading

the sample, the column was washed with several bed volumes of TE buffer. After preparation of the column, the sample was mixed with 2 µl of dye (croecin orange and blue dextran) and applied to the column. The smaller unincorporated nucleotides remained at the top of the column with the orange dye whereas the larger pieces of DNA were eluted with the blue dye. The eluant with the blue dextran dye was collected in fresh Eppendorf tubes and the amount of radioactivity present was determined by analyzing 1 µl of the sample in the scintillation counter.

Hybridization

The nylon membrane containing the immobilised DNA was transferred to a Seal-O-Meal bag and 25 ml of prewarmed prehybridization solution (65 °C) of the following composition was added: 6.25 ml of 20x SSC (5x final conc); 1.25 ml of 100x Denhardt's solution (5x final conc) (2% w/v each of bovine serum albumin, Ficoll and polyvinyl-pyrrolidone); 1.25 ml of 10% (w/v) SDS; 0.5 ml of denatured salmon sperm DNA (10 mg/ml) heated to 100 °C for 5 min; and sterile Milli-Q water to make up the volume to 25 ml.

For pre-hybridization, the membrane was incubated with gentle shaking at 65 °C for 2 to 4 h. The labeled DNA probe (up to 2 x 10^6 counts per min) was denatured (by heating at 100 °C for 5 min) and introduced in the same pre-hybridization solution. Hybridization was allowed to proceed for 14-16 h at 65 °C. The nylon membrane was removed from the bag and transferred to a plastic box for washing. For high stringency, the membrane was incubated twice with 2x SSC containing 0.1% SDS for 10 min at room temperature. This was followed by a wash in 1x SSC containing 0.1% SDS at 65 °C for 15 min and a final wash in 0.1x SSC containing 0.1% SDS at 65 °C for 10 min. For a low stringency wash, the membrane was incubated twice in 5x SSC containing 0.1% SDS solution for 15 min at room temperature followed by a wash in 2x SSC, containing 0.1% SDS for 15 min at room temperature. After washing was complete, the membrane was covered with Saran wrap and autoradiographed. For reprobing the membrane with a different probe, the membrane was stripped by placing it in boiling 0.1% SDS and then cooling it to room temperature. The membrane was again pre-hybridized before hybridization with another probe.

Autoradiography

The membrane was exposed to X-ray film (Kodak X-OMAT AR) in an X-ray cassette with an intensifying screen at -70 °C for 6-16 h, the exposure time varied

depending on the specific activity of the probe. The film was developed using a FUJI RGII X-RAY film processor.

2.19 RNA isolation and analysis

Preparations for RNA work

All the solutions used for RNA work (except for Tris containing buffers) were treated with DEPC (Diethyl pyro carbonate, Sigma Chemicals) solution to a final concentration of 0.1%, for at least 12 h at room temperature and thereafter autoclaved. The tips, tubes and solutions were used only for RNA work, gloves were worn when handling RNA samples. The electrophoresis tank was cleaned with detergent solution, rinsed with Milli-Q water, dried with ethanol and then filled with 3% solution of hydrogen peroxide. After 10 min at room temperature, the tank was rinsed thoroughly with DEPC-treated water and thereafter used only for running RNA samples.

Protocol for RNA isolation

An identical procedure was followed for *E. coli* and *A. vinelandii* RNA isolation. The cells were grown to a required growth stage and then harvested for RNA isolation: 4 x 1.0 ml cells were centrifuged in the cold in a microcentrifuge at 14,000 rpm for 30 sec, the supernatant was poured off and the pellet was immediately resuspended in 400 µl of a phenol/lysis buffer mixture (200 µl of lysis buffer: 0.5 % SDS, 1 mM EDTA in 10 mM Tris-HCl pH 7.0; and 200 µl of neutral phenol mixed together just before use). The tubes were vigorously shaken using a vortex mixer and incubated at 65 °C for 10 min, with intermittent shaking every 2 min, and then centrifuged in a microcentrifuge at 14,000 rpm for 15 min in the cold. The upper aqueous phases from the four identical samples were transferred to fresh tube, pooled and subjected to a phenol:chloroform extraction. The RNA present in the aqueous phase was precipitated on ice for 30 min using 1/10 volume of DEPC-treated 3 M sodium acetate and 2.5 volumes of 95 % ethanol. The RNA was then collecte by centrifuging for 15 min at 4 ^{0}C. The ethanol was decanted and the RNA pellet was air dried. Any DNA present in the RNA sample was removed by dissolving the pellet in 180 µl of DEPC-treated water and 20 µl of 10x DNase buffer (1 M sodium acetate, 50 mM $MgSO_4$, pH 5.0). Next, 2 µl of RNase free DNase (10 units/µl) was added and the tube was incubated at 30 °C for 30 min. This was followed by two phenol:chloroform extractions. One tenth volume of sodium

acetate and 2.5 volumes of 95% ethanol were added to the final aqueous phase containing the RNA. This solution was stored at -70 °C.

Analysis of RNA

Normal agarose gel electrophoresis was used to analyze RNA samples, except that autoclaved TAE buffer was used. To check for possible degradation, the RNA samples were always electrophoresed on an agarose gel before being used in Northern analysis or promoter mapping.

To quantify the amount of RNA present in the sample the absorbance at 260 nm was measured (absorbance of 1 at 260 nm = 40 µg/ml RNA).

2.20 Northern hybridization

Sample preparation

The procedure as described by Williams & Mason (1985) was used for preparing RNA samples for Northern hybridization. The RNA pellet (containing 30 to 40 µg of RNA) was thoroughly resuspended in 2.5 µl DEPC-treated water. Then 2 µl of 40% Glyoxal (de-ionized), 1.5 µl of 80 mM sodium phosphate buffer (pH 6.5) and 6 µl of DMSO were added. The tubes were heated at 50 °C for 1 h and then chilled on ice for 5-10 min. Three microliters of loading dye was added before loading the denatured RNA samples onto the gel. The samples were electrophoresed on a 1.25% agarose gel with a recirculating 10 mM sodium phosphate buffer system at 55 V for about 4 h.

Northern blotting

After the gel was electrophoresed, the RNA was directly transferred onto a nylon membrane using the procedure described for Southern blotting (2.18). The gel was neither stained nor treated with any solution before the transfer. The capillary blot was performed overnight. After the transfer, the membrane was dried and baked in an oven at 80 °C for 2 h.

Labeling of the probe

DNA probes were labeled in two different ways: one was end-labeled and the other was random primer-labeled. Random primer-labeling was done as described for the probe for Southern hybridization (2.18). End-labeling of the probe was done using the following method:

DNA	3 µl (50 picomoles final)
10x Kinase buffer (Boehringer)	1 µl
γ^{32}P ATP 10 µCi/µl	5 µl (50 µCi)
Polynucleotide kinase 10 units/µl (Boehringer)	1 µl

A total 10 µl of labeling reaction was incubated at 37 °C for 30 min, 1 µl aliquot of kinase was added, and the reaction was allowed to proceed for another 30 min.

To remove unincorporated ^{32}P, the reaction mixture was passed through a NucTrap® probe purification column (Stratagene). To quantify the amount of radioactivity in the probe, 1 µl of the sample was analyzed in the scintillation counter. For Northern hybridizations, probes with $2x10^6$ - $5x10^6$ cpm were used.

Hybridization

When PCR1 was used as a probe, 50% formamide hybridization solution of the following composition was used:

20x SSC	1.5 ml (final concn. 3x)
100x Denhardt solution	0.4 ml (final concn. 4x)
Deionized formamide	5 ml
Denatured salmon sperm DNA	0.1 ml (stock solution of 10 mg/ml)
Sterile Milli-Q water	3.0 ml

The nylon membrane with immobilized RNA was prehybridised with the non-homologous DNA at 45 °C for 2 h. The hybridization was carried out at the same temperature for 12-16 h after addition of $5x10^6$ cpm of probe. For calculating the Tm of the PCR1 probe for hybridization, the following equation was used:

$$Tm = 81.5\ ^0C + 16.6\ (\log Na^+) + 0.41\ (\%\ G+C) - 500/L - 0.61\ (\%\ \text{formamide})$$

where L is the length of the probe (286 bp), Na^+ concentration was 0.4 M in 3x SSC, and the formamide used was 50%. The Tm calculated according to this equation was 50 °C. Therefore, pre-hybridization and hybridization was done at Tm – 5 °C.

For the oligonucleotide probe, the hybridization procedure was the same as that used in Southern hybridization, except that the temperature at which the pre-hybridization and hybridization was carried out was 50 °C. For calculating the Tm of the oligonucleotide probe for hybridization, the following equation was used:

$$Tm = 81.5\ ^0C + 16.6\ \log_{10}(Na^+) + 0.41\ (\%\ G+C) - 820/L$$

where (Na^+) is the concentration of sodium ions in the solution (0.75 M in 5x SSC) and L is the length of the probe (17 bases). The Tm calculated according to this equation was 60 °C. Therefore the temperature at which prehybridization and hybridization was carried out was at 10 °C below the Tm.

Following hybridization, the membrane was washed twice with 2x SSC containing 0.1% SDS at room temperature for 15 min and again in the same solution at 45 °C for 15 min. After washing, the membrane was wrapped in Saran wrap and exposed to X-ray film or a phosphor screen. The phosphorimager files were imported to Adobe Photoshop (for Power Macintosh) programme to optimize the contrast and background.

2.21 Promoter mapping studies

2.21.1 S1 nuclease analysis

S1 nuclease analysis was done according to the method described by Hopwood *et al.* (1985) with slight modifications.

PCR1 was end-labeled with γ ^{32}P ATP (Section 2.20) and used as a probe for S1 analysis. RNA (50 µg) from each sample was combined with 1 µl of the probe (50,000 cpm). The mixture was dissolved in 20 µl of S1 hybridization solution (40 mM PIPES pH 6.4 containing 400 mM NaCl, 1 mM EDTA and 80% v/v formamide). The tubes were incubated at 85 °C for 30 min with mixing at 15 min. The waterbath was adjusted to 50 °C and the tubes were allowed to cool down slowly to the annealing temperature of 50 °C. After annealing overnight, the samples were transferred to ice and 300 µl of S1 digestion solution (60 µl 5x S1 buffer and 240 µl DEPC-treated water containing 150 units of S1 nuclease enzyme; Boehringer) was added. The solution was mixed well and incubated at 37 °C for 45 min. The tubes were then transferred to ice and S1 nuclease digestion was stopped with the addition of 75 µl of the S1 termination solution. Next, the S1 nuclease protected, RNA-DNA hybrid fragments were precipitated by adding 2 µl glycogen (Boehringer) and 400 µl of 95% ethanol. The tubes were incubated at -20 °C for about 1 h. The pellet was collected by centrifugation and washed once with 95% ethanol before dissolving in 3 µl of stop solution (from the Sequenase® sequencing kit). The samples were analyzed on a 6% polyacrylamide sequencing gel. Random primer-labeled DNA molecular weight marker # III (Boehringer) was used as a size standard.

2.21.2 Primer extension analysis

Primer extension analysis was performed as described by Ausubel *et al.* (1992) with some modifications. The 17-mer, WJP1 end-labeled with ^{32}P, was used as the primer. Forty micrograms of RNA from each sample was pelleted and dried. The RNA pellet was dissolved in 10 µl of aqueous hybridization buffer (containing 3 M NaCl, 0.5 M Hepes pH 7.5 and 1 mM EDTA pH 8.0), about $6x10^5$ cpm end-labeled probe was added, and the total volume was made up to 30 µl with DEPC-treated water. Nucleic acid mixtures were heat-denatured (85 °C for 5 min) prior to annealing for one hour at 42 °C. Annealed nucleic acid mixtures were precipitated with 2.5 volumes of ethanol (tubes were left on dry ice for 45 min). Thereafter, 25 µl of reverse transcriptase mix (consisting of: 7 µl of 2 mM dNTP, 5 µl of 5x reverse transcriptase buffer (Boehringer) and 0.5 µl RNase inhibitor (Boehringer)) were added to the dry pellet. Adding 25 units of AMV reverse transcriptase (Avian Myeloblastosis Virus reverse transcriptase, Boehringer) and then incubating the tubes at 42 °C for 1 h facilitated the extension of the bound primer. Nucleic acid mixtures were precipitated with 1 µl of 20 mg/ml glycogen (Boehringer), 30 µl of 3 M sodium acetate, 243 µl TE buffer and 2.5 volumes of ethanol. The tubes were centrifuged in the cold and the pellet was dissolved in 8 µl of the stop solution from a Sequenase version 2.0 kit (United States Biochemicals). The samples were heat denatured (85 °C for 5 min) before loading on a 6% polyacrylamide sequencing gel.

A sequencing reaction, using WJP1 as the primer and M13 mp19 clone #2 (containing the *Hin*dIII-*Sal*I fragment of pMVW98) as the template, was set up to analyze the 5' end of the gene.

2.22 Western blot analysis

2.22.1 Preparation of cytoplasmic extracts

Cytoplasmic extracts from *A. vinelandii* and *E. coli* cultures were prepared according to the method described by Lam *et al.* (1994). An overnight culture (50 ml) was harvested by centrifugation at 8,000 xg for 5 min. The bacterial cells were then suspended in 5 ml of sonication buffer (10 mM sodium phosphate buffer, 5 mM $MgSO_4$, pH 7.0). The cells were sonicated, using a needle probe (40T, Braun Sonic 2000) with a 60 sec cycle and 40 sec cooling interval. Samples were observed periodically under the microscope to check whether the procedure was successfully completed. DNase I was

added to a final concentration of 30 µg/ml, and the mixture was incubated at 37 °C for 15 min. Unbroken cells were removed by centrifuging the mixture at 6,000 xg for 20 min. The supernatant consisted of the cell envelope and the cytoplasmic fraction. The former was removed by centrifuging the supernatant at very high speed (36,000 rpm in a type 40 ultracentrifuge rotor) for 1 h. Adding 4 volumes of acetone, and incubating the mix at -20 °C for 1 h, precipitated proteins (including Fur) that were present in the supernatant. The tubes were centrifuged at 12,000 xg for 10 min in the cold. The pellet was air-dried at room temperature and resuspended in 0.5 ml of Milli-Q water and 2x SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% β-mercaptoethanol). Protein concentration was determined by the Bradford method (1976). Cytoplasmic extracts were stored at -70 °C until used. The extracts were boiled for 5 min. Any insoluble material was removed by centrifugation before examination by gel electrophoresis.

2.22.2 SDS polyacrylamide gel electrophoresis of proteins

Proteins present in the cytoplasmic extract were separated on a 15% SDS -polyacrylamide gel. Approximately 20 to 100 µg of protein was loaded into each of the lanes. Pre-stained markers (Gibco-BRL) in the range of 15 kDa to 200 kDa were used as size standards.

A mini-gel protein apparatus (Mighty Small II, Hoefer Scientific Instruments) was used. The 15% resolving gel was prepared as follows:

Acrylamide bis-acrylamide solution 40% (29:1)	3.75 ml
1.5 M Tris-HCl, pH 8.8	2.6 ml
10% SDS	0.05 ml
10% Ammonium persulfate	0.05 ml
TEMED	0.004 ml
Milli-Q water	to 10.0 ml

After the resolving gel was set, 5% stacking gel was made. The composition of stacking gel (total volume 4 ml) was as follows:

Acrylamide bis-acrylamide 40% (29:1)	0.5 ml
1.0 M Tris-HCl, pH 6.8	0.5 ml
10% SDS	0.04 ml
10% Ammonium persulfate	0.04 ml
TEMED	0.004 ml

Milli-Q water to 4.0 ml

The gel running buffer was Tris-Glycine (per liter: 3.0275 g Tris, 14.413 g Glycine and 1 g SDS). Electrophoresis was performed, first at 15 milliamps (per gel) through the stacking gel and then at 25 milliamps through the resolving gel, until the blue dye reached the bottom of the gel. Two gels with identical samples were prepared in all case, so that one gel could be stained with Coomassie blue stain (0.5 g coomassie blue R250 in 90 ml water, 90 ml methanol and 20 ml acetic acid) while the other was used for Western transfer.

2.22.3 Western transfer

For transferring the proteins from the gel to the nylon membrane, a Bio-Rad mini-Transblot apparatus was used.

After electrophoresis, the stacking gel was removed and the resolving gel was transferred to a clean plastic dish. The latter was equilibrated with the transfer buffer (12 mM Tris-HCl pH 9.0, 96 mM glycine and 20% methanol) for 15 min. Four pieces of Whatman 3MM paper and a piece of nylon membrane were cut to the size of the gel and presoaked in the transfer buffer. The gel was transferred to two wet Whatman paper sheets laid over the foam on the black plate (anode). A glass pipette was rolled over the surface of the gel to remove bubbles and a nylon membrane was placed over the gel without introducing any air bubbles. The two sheets of Whatman paper were placed on top. The cathode (white plate) was placed over this stack and the latches were carefully engaged with the guide pins in the corners of the apparatus. The chamber of the box was filled with the transfer buffer. After replacing the safety cover, the wires were connected to proper outlets (transfer polarity being from cathode to anode).

The transfer was done in the cold at 200-milliamps constant current for 1 h. The efficiency of the transfer was checked by observing the transfer of the pre-stained markers (Boehringer).

2.22.4 Immunodetection

After transfer, the membrane was placed in a plastic dish of the size of the filter, and washed 5 times 2-3 min each with 50 ml TBS (50 mM Tris-HCl, pH 8.0 containing 150 mM NaCl). It was then blocked with 50 ml TTBS (TBS with 0.1% (v/v) Tween 20)

containing 4% (v/v) bovine serum albumin for at least 2 h at room temperature (or overnight at 4 °C) and washed twice for 10 min with 50 ml TTBS-0.5% BSA. The membrane was then incubated for at least 2 h in 30 ml of primary antibody solution. The primary antibodies used were rabbit, anti-Fur (*E. coli*) and anti-Fur (*P. aeruginosa*) and were diluted (1:300 to 1:500) in 30 ml of TTBS-0.5% BSA. After primary antibody binding, the membrane was washed 4 times for 5 min each with 50 ml TTBS-0.5% BSA and incubated in 30 ml of diluted secondary antibody for approximately 2 h. The secondary antibody used was anti-rabbit IgG-alkaline phosphatase conjugate (500 Units, Boehringer), which was diluted 1:7000 in 30 ml TTBS-0.5% BSA. The membrane was washed 4 times for 5 min each in 50 ml TTBS and rinsed in AP buffer (0.1 M Tris-HCl pH 9.5 containing 0.1 M NaCl and 5 mM $MgCl_2$) once. The color was developed by adding 30 ml of freshly prepared alkaline phosphatase substrate solution containing 0.2 ml of 50 mg/ml NBT (nitro-blue tetrazolium, made in 70% v/v dimethyl formamide) and 0.1 ml of 50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate-p-toluidinium salt, made in dimethyl formamide) in 30 ml of AP buffer. The membrane was incubated in this solution for 2-10 min until the intensity of the band was bright enough to be visible clearly. The AP substrate solution was protected from light and used within 1 h. The color reaction was stopped by adding 50 ml of AP stop solution (20 mM Tris-HCl, pH 8.0 with 20 mM EDTA). The membrane was rinsed with Milli-Q water and air dried on Whatman paper in the dark, sealed in Saran wrap, and stored in the dark.

2.23 Electroporation in *A. vinelandii* UW and VK20 strains

2.23.1 Preparation of electrocompetent cells

A. vinelandii strains UW and VK20 were maintained on slants of Burk's medium at room temperature. *E. coli* strains harboring the plasmids of interest were grown on LB agar plates supplemented with appropriate antibiotics. For plasmid extraction, the strains were grown in LB (supplemented with appropriate antibiotic) medium overnight at 37 °C with shaking. Plasmids were extracted (section 2.6) and washed thoroughly with 70% ethanol to remove the salts before resuspending in sterile water.

A. vinelandii strains were grown on BBGN plates for 2-4 days at 28 °C to get isolated colonies. A few colonies were used to inoculate a starter culture (20 ml BBGN in 50 ml flasks). The flasks were shaken overnight at 28 °C and 175 rpm. Next, 100 ml of BBGN was inoculated with 1 ml of the overnight culture. The cells were allowed to

grow to $O.D_{600}$ of 0.5 to 0.7, chilled on ice for a few minutes, and harvested by centrifugation at 4,000 xg for 5 minutes at 4 ^{0}C. Thereafter, the cells were washed 3-4 times with 0.1 M glucose. After the last wash, the cells were resuspended in 1.5 ml of 15% glycerol. The solution was transferred to an Eppendorf tube and centrifuged in a microcentrifuge at 6,500 rpm for 5 min. The cells were finally suspended in 500 µl of 15% glycerol and used in the electroporation experiment within a few hours. To electroporate approximately 100 ng of DNA, 40 µl of electrocompetent cells were used.

2.24 Phenylalanine t-RNA synthetase assay

2.24.1 Preparation of S-160 extracts

S-160 extract was prepared by a modified method of Comer and Bock, 1976. Three day old slants of *A. vinelandii* UW were used to inoculate the starter cultures (20 ml in a 50 ml flask) in OFeBBGN + 1 µM ferric citrate (low-iron) and in OFeBBGN + 100 µM ferric citrate (high-iron). The starter cultures were grown for 20-24 h and used to inoculate their respective 2 liter culture flasks at 4% (v/v) for the low-iron medium (600 ml in a 2 liter flask) and 2% (v/v) for the high-iron medium (500 ml in a 2 liter flask). The low-iron culture was allowed to grow for about 18 to 19 h to obtain an OD_{600} of 1, while the high-iron culture was grown for 15 to 16 h to reach the same OD_{600}. The cells were harvested by centrifugation at 8,000 xg for 10 min and washed once in the following wash buffer:

Tris-HCl, pH 7.5	20 mM
Magnesium acetate	10 mM
Ammonium chloride	30 mM
Dithiothreitol (DTT)	2 mM
Ethylenedinitrilotetra acetic acid	0.2 mM

After one wash, the cells were resuspended in the same buffer (5 ml/g of wet cells) and DNase was added to a final concentration of 5 µg/ml. The cells were broken by two passes through a French pressure cell (French Pressure Cell Press, AMINCO) and observed using the microscope to confirm the success of the process. Cell debris, along with unbroken cells, was removed by centrifuging the mixture at 30,000 xg for 30 min at 4 ^{0}C. The supernatant thus obtained was transferred to a Beckmann polycarbonate tube suitable for use in an ultracentrifuge type 50 Ti rotor. The tubes were centrifuged for 2 h at 45,000 rpm (160,000 xg) at 4 ^{0}C in a Beckmann Ultracentrifuge. The supernatant thus

obtained was S-160 extract and was either used immediately or stored at –70 ^{0}C (with 15% glycerol).

The resulting S-160 extract contained 4 to 10 mg of protein per ml, as determined by the Lowry method (Lowry *et al.*, 1951).

2.24.2 PheST assay

Two duplicate reaction mixtures (100 µl each) were setup as follows:

Tris-HCl pH 7.5	50 mM
DTT	1 mM
Magnesium acetate	10 mM
ATP	2 mM
t-RNA	1 mg
^{14}C-Phenylalanine	1 µCi

The reaction was started by adding 20 µl of S-160 extract from the low-iron or high-iron cultures. A 20 µl aliquot of sample was withdrawn at the beginning, immediately transferred to a 1.5 cm^2 Whatman #1 filter paper, and dropped in a flask containing chilled 5% trichloroacetic acid (TCA), to stop the reaction. Thereafter, 20 µl samples were withdrawn at 2, 4, 8 and 16 min, similarly loaded onto 1.5 cm^2 filter paper, and dropped into flasks containing chilled TCA. The filters were washed three times in chilled 5% TCA for about 15 min with gentle shaking, followed by a single wash in chilled ethanol:diethylether (50:50) solution for 10 min, and once in diethylether for 5-10 min. The filter papers were air-dried for 10 to 15 min and transferred to scintillation counter vials (Fisher Scientific co) containing 8 ml of scintillation fluid (Aquasol-2, NEN Research Products). Disintegration per min (dpm) was measured using a scintillation counter (Beckman LS 3801).

The value in dpm was converted into pmols/ml. Since, the specific activity of ^{14}C-phenylalanine was 513 mCi/mmol, each reaction contained 1 µCi of the same. The 20 µl of reaction mixture (loaded on to the filter paper) contained 0.2 µCi ^{14}C-phenylalanine, which was equivalent to 0.39 nmols of ^{14}C-phenylalanine in each reaction mixture. The enzyme activity was calculated in terms of pmols/ml of the reaction mixture at each time point. Total protein concentration was calculated in terms of µg per ml.

Chapter 3
Results

3.1 Phenotype of Strain UA22

3.1.1 Preliminary studies

UA22 is a Kan^R Lux-positive mutant, previously constructed by insertional mutagenesis of wild-type strain UW with pTn*5luxAB* (Sevinc & Page, 1992), whose phenotype has not been previously described. Initial work by Dr. M.V. Woestyne showed that bioluminescence of one or two-day plate culture was strong in low-iron (1 μM Fe^{+3}) medium and only faintly present in high-iron (300 μM Fe^{+3}) medium. Siderophore production by strain UA22 was repressed in liquid Fe-sufficient (20 μM Fe^{+3}) medium and derepressed in Fe-limited (1 μM Fe^{+3}) medium, following the sequential derepression pattern obtained in other studies with the wild-type (Page & Huyer, 1984; Page & von Tigerstrom, 1988). The first appearance of bioluminescence in Fe-limited medium was observed at 15 hr, at the beginning of the stationary phase of growth and near the onset of azotobactin production (Woestyne & Page, unpublished results). In fact, strain UA22 always appeared to form less azotobactin (observed as a yellow green coloration in the medium) than strain UW. Hence, it was believed that the mutation in UA22 might influence azotobactin production.

3.1.2 Siderophore production by strain UA22

In order to study the phenotype of UA22 with respect to the siderophore production, yields of catechols and azotobactin were measured and compared with that of the wild type. Two sets of OFeBBGN medium were prepared and supplemented with 1 μM ferric citrate or 0.5 mg/ml micaceous hematite. Micaceous hematite has previously been shown to cause the hyperproduction of azotobactin when added to a Fe-limited culture of *A. vinelandii* (Page & Huyer, 1984). The experiment was repeated three to four times and the results in Table 3-1 show a representation of those results. There was a 1.6 to 2 fold increase in azotobactin production by the strains UW or UA22 when micaceous hematite was present as compared to siderophore production in the presence of 1 μM ferric citrate. However, when UA22 was grown in presence of kanamycin, the antibiotic used to select and maintain this strain, azotobactin production dropped 2 to 4.5 fold with either iron source. Catechol production was affected less by the presence of micaceous hematite or kanamycin.

Strain	Form of Iron	Presence of kanamycin	Catechols (A310/ mg protein)	Azotobactin (A380/mg protein)
UW	1 µM Ferric citrate	-	3.4	3.0
UA22	1 µM Ferric citrate	-	3.3	2.7
UA22	1 µM Ferric citrate	+	1.9	0.6
UW	Micaceous hematite	-	4.3	6.1
UA22	Micaceous hematite	-	4.6	4.3
UA22	Micaceous hematite	+	3.6	2.2

Table 3-1 Comparison of the yield of catechols and azotobactin siderophores produced by UW and UA22. Samples were withdrawn after 24 h for analysis of siderophores and for protein assay.

3.1.3 Purification of azotobactin from UW and UW2

Since the mutant strain UA22 did show some difference in levels of azotobactin produced as compared to the wild type strain, UW, azotobactin was purified from both the strains and analyzed by Fast Atomic Bombardment (FAB) mass spectroscopy[1].

The results of FAB-mass spectroscopy are shown in figures 3-1 and 3-2. Azotobactin from both strains shared the three major fragments with molecular weights 588, 808, and 417 characteristic of this siderophore. The fragment at 808 is the peptide arm of azotobactin without the first three amino acids which remains attached to the chromophore. The fragment at 588 corresponds to the chromophore moiety with attached amino acids aspartate, serine, and homoserine while the fragment at 417 corresponds to the chromophore with an attached aspartate residue. However, the FAB-mass spectra also indicated differences between the UA22 and UW azotobactin preparations, possibly because these were not completely pure.

3.1.4 Effect of kanamycin on azotobactin production

To check whether the decreased production of azotobactin seen in Table 3-1 was typical for strain UA22, four other Tn*5 luxAB* mutants of *A. vinelandii* (D13, D48, E21, F196) were grown in OFeBBGN with different amounts of kanamycin. Strain D13 was found to produce luminescence constitutively, D48 was reported to be a catechol overproducer, mutant E21 was reported to overproduce azotobactin and F196 was reported to be a catechol-negative mutant. The results shown in Table 3-2 imply that kanamycin has a negative affect on the production of azotobactin in general. Therefore, UA22 is not unique in its response to kanamycin and it cannot be concluded that it carries a defect in azotobactin production.

[1] Department of Chemistry Service, University of Alberta

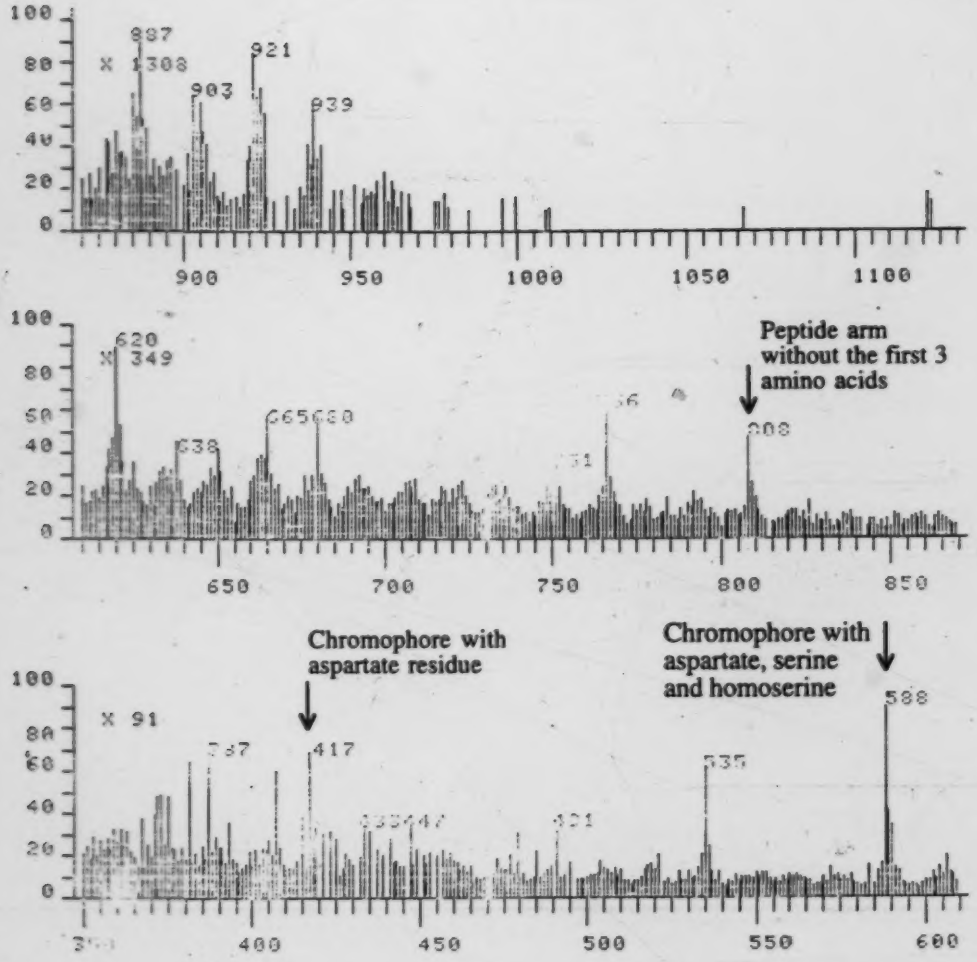
Peptide arm without the first 3 amino acids
Chromophore with aspartate residue
Chromophore with aspartate, serine and homoserine

Fig. 3-1 Fast Atomic Bombardment mass spectrometry spectrum of azotobactin isolated from *A. vinelandii* wild type strain UW. The arrows point to the major peaks of the breakdown products of azotobactin.

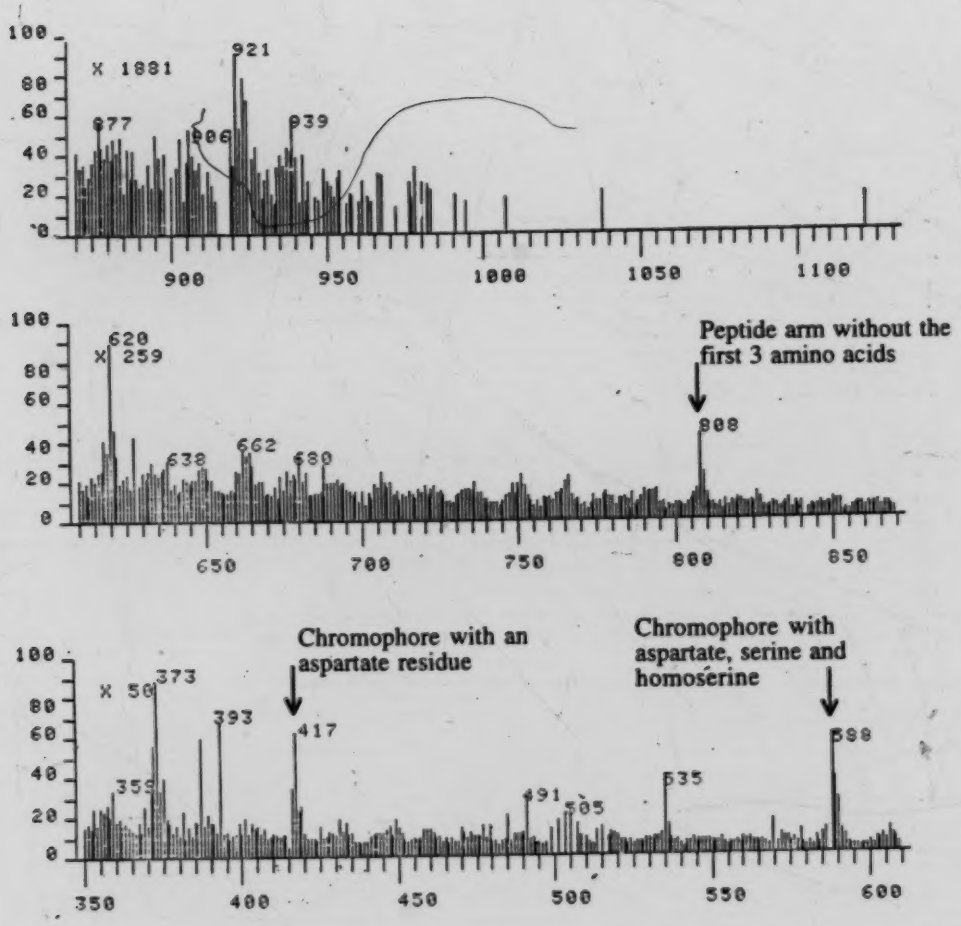

Fig. 3-2 Fast Atomic Bombardment mass spectrometry spectrum of azotobactin purified from *A. vinelandii* strain UA22. Three major peaks of azotobactin, as seen with the azotobactin from the wild type strain, are shown with arrows.

Strain	Concentration of kanamycin (μg/ml)	Catechols (A310/mg protein)	Azotobactin (A380/mg protein)
UW	0	3.7	0.6
D13	0	3.2	0.5
	5	3.2	0.7
	10	2.7	0.6
	15	2.9	0.5
D48	0	3.7	0.7
	5	3.0	0.3
	10	2.9	0.6
	15	2.8	0.2
E21	0	1.0	2.9
	5	0.8	1.4
	10	0.8	0.7
	15	0.9	0.5
F196	0	0	2.6
	5	0	1.0
	10	0	0.6
	15	0	0.4
UA22	0	3.8	0.5
	5	3.0	0.3
	10	2.6	0.3
	15	2.5	0.2

Table 3-2 Effect of kanamycin on siderophore production by different Tn*5* mutant strains of *A. vinelandii*.

3.2 Sequencing of UA22 DNA

3.2.1 Preliminary work[2]

In an effort to identify the inactivated gene in *A. vinelandii* UA22, the genomic DNA of strain UA22 was isolated and screened for the presence of the Tn*5* insert. A cosmid library of UA22 genomic DNA was made in pLAFR3 and transduced into *E. coli* VCS257. Cosmid containing clones were selected on LB-Tet medium and screened for kanamycin resistance (indicating the insertion of Tn*5*). Among the thousands of Kan^R isolates, only one clone (pMVW31) demonstrated Fe-repressible Lux activity. The cosmid pMVW31 was subcloned in several steps, and the smallest subclone (3.2 kb *Sma*I-*Cla*I fragment in pUC119) which demonstrated Fe-repressible Lux activity, but was sensitive to kanamycin, was pMVW97. From the map of *luxAB* genes (Cohn *et al*, 1985), it was determined that there is a *Pst*I site 222 bases from the start of the *luxA* gene. Therefore, the iron-regulated promoter should be located in the 1.3 kb *Pst*I-*Sma*I fragment of pMVW97. This fragment was subcloned into the *Pst*I-*Sma*I site of pUC119 to construct pMVW98 (Fig. 3-3).

3.2.2 Subcloning in M13 phage vectors

In order to identify the iron-regulated promoter and the gene disrupted by the insertion of *luxAB*, the nucleotide sequence of the 1.3 kb *Pst*I-*Sma*I in pMVW98 needed to be determined. Since, *Azotobacter* DNA is rich in G+C bases (mol% G+C is 65-67%), single stranded DNA sequencing using clones in M13 phage vectors was used.

On digesting pMVW98 with *Hind*III and *Sal*I, two fragments were obtained besides the vector: a smaller (≈ 300 bp) and a larger (≈ 650 bp) *Hin*dIII-*Sal*I fragment (Fig. 3-3). Both fragments were individually ligated to the *Hin*dIII-*Sal*I digested M13 phage vectors mp18 and mp19. The *Sma*I-*Hin*dIII fragment of pMVW98 was also subcloned into mp18 and mp19 phage vectors. The ligation mixtures were used to transfect *E. coli* DH5α F cells. Positive (white) plaques were identified. Single stranded DNA derived from these plaques was used as template for sequencing.

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[2] Done by Dr. Marleen Vande Woestyne

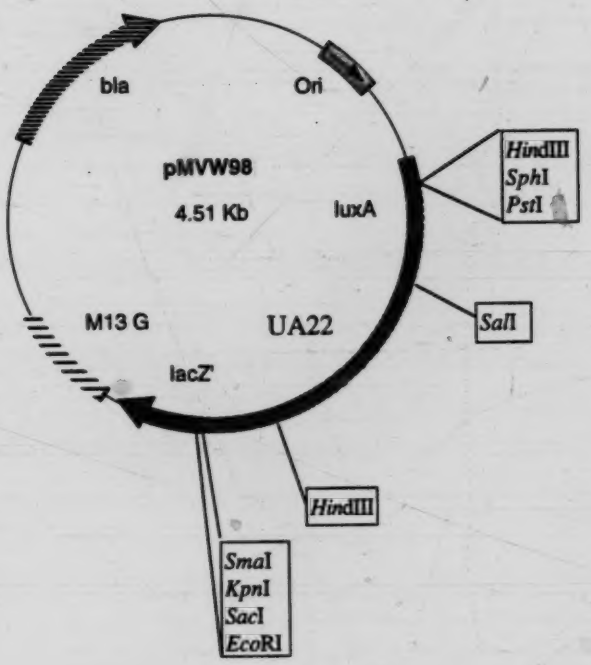


Fig. 3-3 Schematic of plasmid pMVW98 showing the position of the 1.3 kb *Pst*I-*Sma*I fragment of *A. vinelandii* UA22 DNA. Restriction enzyme sites of interest are noted.

3.2.3 Sequencing strategy

Clone #1 contained the larger *Hin*dIII-*Sal*I fragment. First, mp18 clone #1 was sequenced using the universal primer and a 458 base sequence was obtained. When mp19 clone #1 was sequenced (to obtain the sequence in the other direction) using the universal primer, a 401 base sequence was obtained obtained which overlapped the former sequence by 227 bases. To confirm the rest of the sequence, two synthetic oligonucleotide primers were synthesized. Primer WJP1 was used with the mp19 clone #1 while primer WJP2 was used with the mp18 clone #1 (Table 3-3). The entire sequence of the *Hin*dIII-*Sal*I fragment was found to be 635 bases long (including the restriction enzyme sites).

Next, the smaller *Sal*I-*Hin*dIII fragment (clone#2) was sequenced, using the universal primer and mp19 or mp18 clone #2 as the template, to get the sequence in both directions. A sequence of 407 bases was obtained in both directions with a complete overlap. The sequence length of the *Sal*I-*Hin*dIII fragment was found to be 377 bases (restriction enzyme sites inclusive). The size of the *Sal*I-*Pst*I fragment within this sequence was 365 bp.

Primer WJP3 (Table 3-3) was synthesized to sequence the *Sal*I junction (automated DNA sequencing) using pMVW94 as the template (Fig. 3-4). The *Sma*I-*Hin*dIII fragment in mp18 and mp19 (clone# 3) vectors was then sequenced using the universal primer. The complete sequence was found to be 324 bases (restriction enzyme sites inclusive). Primer WJP4 was synthesized to sequence the *Hin*dIII junction (automated DNA sequencing), also using pMVW94 as the template (Fig. 3-4).

On compiling all the sequences together, the total DNA sequence of the *Sma*I-*Pst*I fragment of pMVW98 was found to be 1313 bp long.

3.2.4 DNA sequence analysis and gene bank searches

In order to ascertain the identity of the gene disrupted by the insertion of Tn*5luxAB*, the nucleotide sequence of the 1313 bp *Pst*I-*Sma*I fragment in pMVW98 was analyzed using the BLAST program for homology search in the gene bank data base. The initial sequence comparisons indicated the presence of an ORF, with 63% identity at the DNA level and 66% identity at the amino acid level, with the *E. coli pheS* gene, which encodes the alpha-subunit of phenylalanyl-tRNA synthetase (Fayat *et al.*, 1983). Since the region

Name	Sequence 5' - 3'	Template	Region of overlap
WJP1	GTGTCGTGCATCGCCCG	M13 mp19 clone #1	Complementary to bases 878 to 861
WJP2	TGCGCACCATGGAAAGT	M13 mp18 clone #1	Bases 931 to 948
WJP3	CCGGACATGCCCAGCAC	pMVW94	Complementary to bases 1292 to 1275
WJP4	ATAGCGAAGACGTCAATGCCC	pMVW94	Bases 457 to 478
WJP5	GGTGACGCTCGGCACCGGGC	pMVW94	Complementary to bases 258 to 238
WJP6	GCAGACAGTGATCAAGGCTG	pMVW94	Upstream of base 1
WJP7	CGTGCCCTGTGGATCGCCCG	pMVW94	Bases 31 to 50

Table 3.3 Synthetic oligonucleotide primers used for sequencing the 1.541 kb UA22 DNA. The sequence of the primers is given 5'---->3' and the positon corresponds to the sequence in Fig. 3-6.

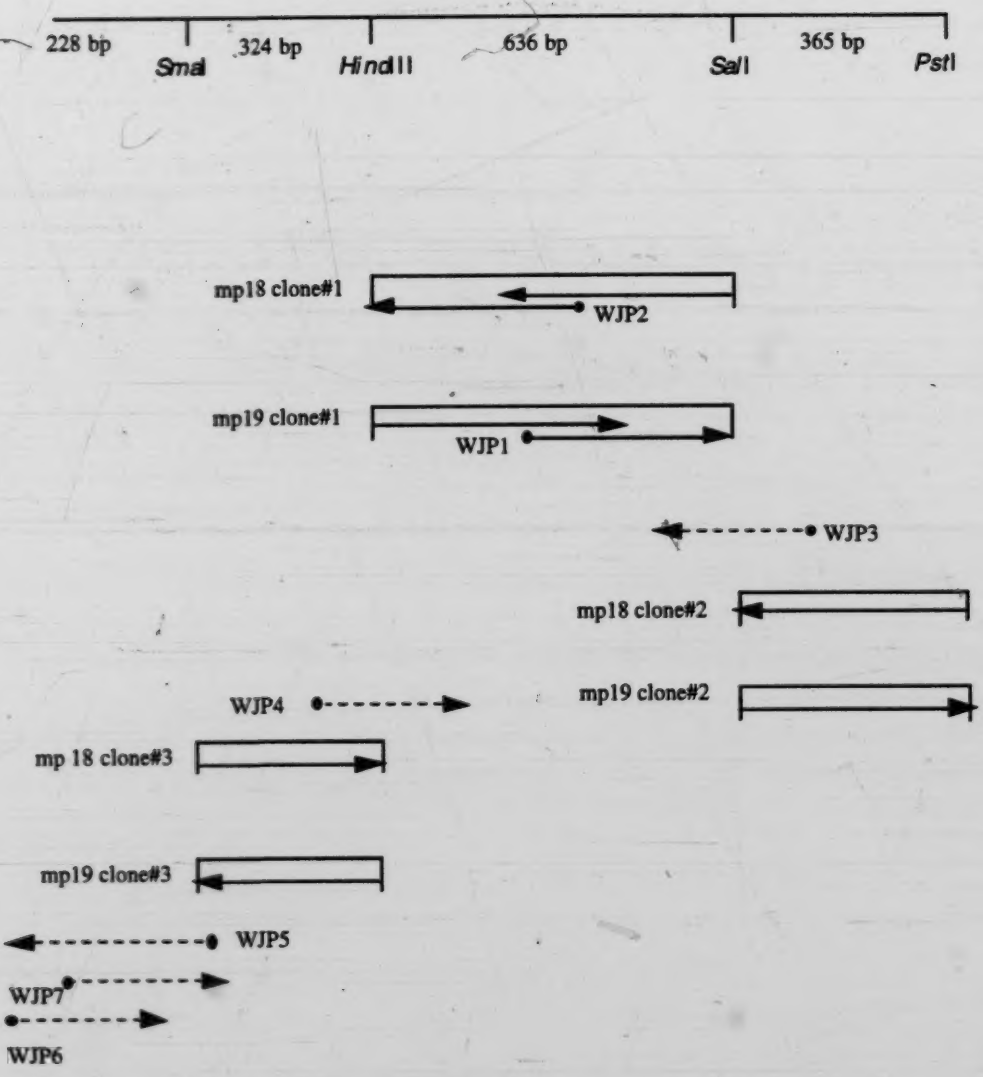


Fig. 3-4 Sequencing strategy. UA22 DNA (1.541 kb) was sequenced in 4 pieces, the subclones in the M13 mp18/mp19 vectors are indicated. Solid arrows originating from a solid vertical bar indicates that a universal primer was used. Solid arrows originating from a • indicates that a synthetic oligonucleotide was used as a primer. The Broken arrow indicates the sequence obtained from the automated sequencer. The size of the 4 fragments are given in bp (base pairs).

upstream of the *E. coli pheS* gene contains the *pheS* promoter sequence (Grunberg-Manago, 1987), examination of sequences upstream of the 1.313 kb *Pst*I-*Sma*I fragment of *A. vinelandii* DNA became very important. Therefore, some extra sequence upstream of the *Sma*I fragment was determined by automated DNA sequencing. The template used was pMVW94. Two more primers were synthesized, WJP5 & WJP6 (Table 3-3). To confirm the sequence obtained by using the above two primers and to overlap the *Sma*I junction, another primer, WJP7, was made. An additional 228 bp of sequence upstream of the *Sma*I site was thus determined. The total length of the sequence of *A. vinelandii* DNA obtained was 1541 bp.

Since *Azotobacter* DNA is normally very GC rich (mol %G+C ≈ 66%), GCG analysis was done on the sequence obtained to see if there was a bias for the G and C's in the sequence and to test for sequencing errors. A codon preference graph was constructed using the *Azotobacter* codon usage table (obtained from the internet site address: http://www.dna.affrc.go.jp/~nakamura/CUTG.html). Fig. 3-5 shows a clear bias for G or C's at the third position in the codons used. Also, there are two open reading frames (ORF's) in the +1 frame, as indicated by the open boxes. The first one ends at position 213 and the second one starts at base 409. There is a frame shift in the region of bases 1303-1309 and a third ORF in the +2 frame, indicated by a drop in the codon preference curve as well the third position GC bias curve. The vertical bars below each frame, depicts the usage of rare codons. Typically rare codons are used less frequently within an open reading frame, and this is in fact seen in Fig. 3-5.

The entire sequence obtained is shown in Fig. 3-6. This sequence was analyzed using the BLAST program for homology searches in the gene bank data base. The results show that nucleotides 1-219 have 72% identity at the DNA level and nucleotides 1-213 have 74% identity at the amino acid level (Fig. 3-7) with the *E. coli rplT* gene (amino acids 47 to 117), which encodes the 50S ribosomal protein L20 (Fayat *et al.*, 1983). This part of the *Azotobacter* DNA was missing the start of the *rplT* gene (first 46 amino acids). Nucleotides 409-1308 showed 63% homology with the *pheS* gene of *E. coli* at the DNA level and 66% at the protein level (Fig. 3-8). Therefore, this ORF is believed to encode the *A. vinelandii pheS* gene, with the possible start codon at position 409 and a likely ribosome binding site (GGAGG) at position 398 (Fig. 3-6). This *pheS* gene is interrupted at its 3' end by the insertion of nucleotides 1309-1541, which are 100% identical to the *luxA* gene of *Vibrio harveyi* (at the DNA level with bases 1303-1541 and at the protein level with bases 1319-1541). The *luxA* start codon is located at position 1319 and ribosome binding site is located at position 1309 (Fig. 3-5 and Fig. 3.6).

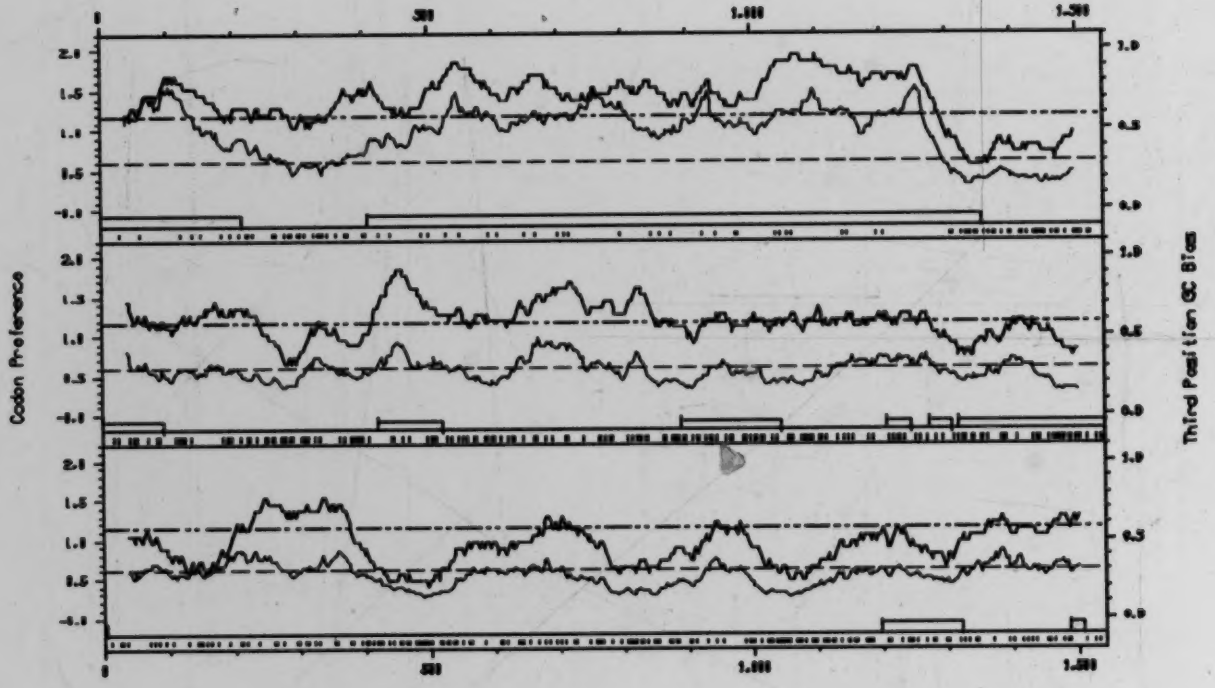
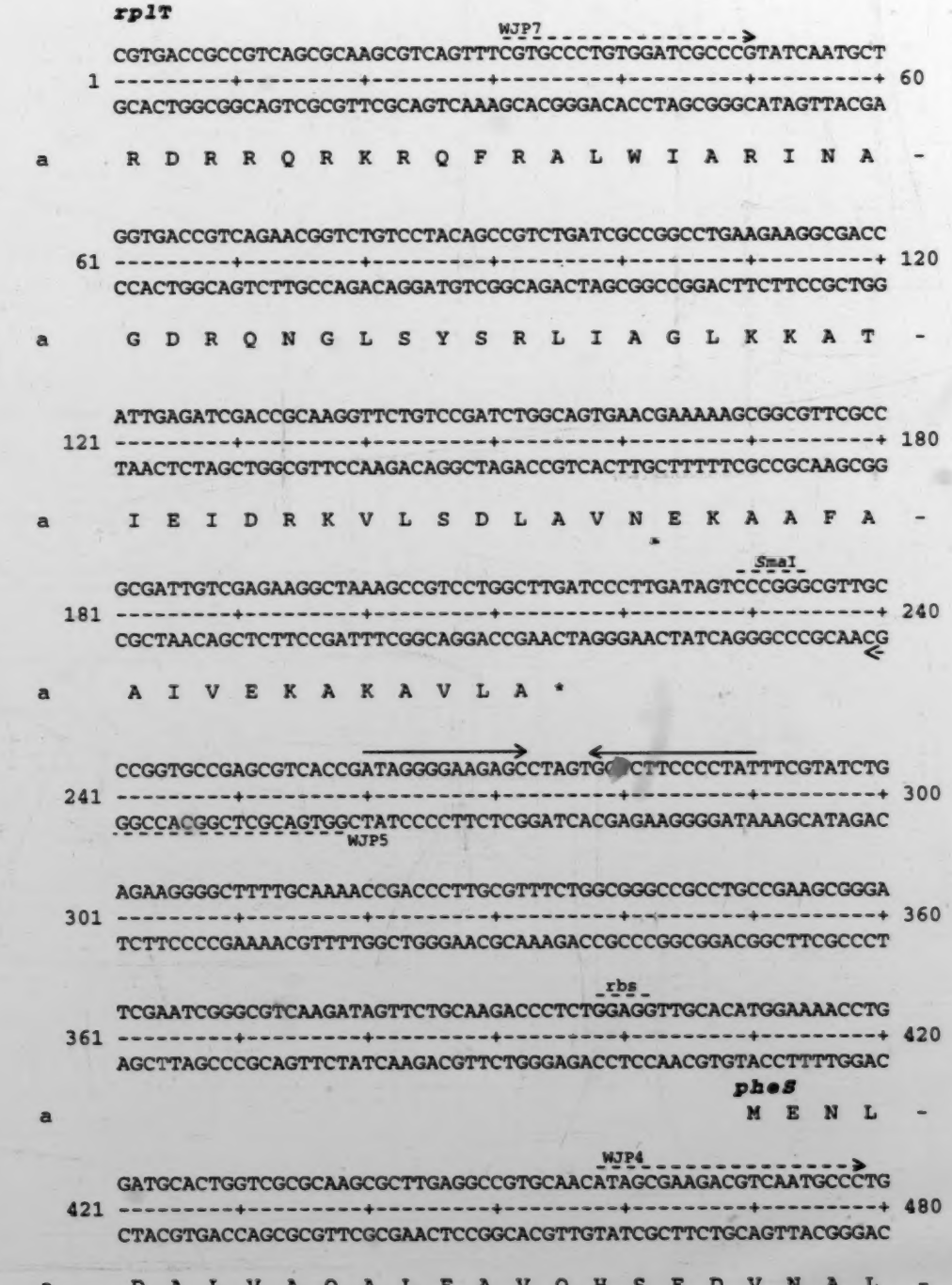


Fig. 3-5 Identification of likely gene coding regions by codon preference with the *A. vinelandii* coding probability plots of the ORF's of the 1.541 kb DNA sequence. Blue line indicates the third position GC bias and the red line indicates the codon preference. The boxes at the bottom of each frame depicts the ORF .


rplT
WJP7
CGTGACCGCCGTCAGCGCAAGCGTCAGTTTCGTGCCCTGTGGATCGCCCGTATCAATGCT
1
GCACTGGCGGCAGTCGCGTTCGCAGTCAAAGCACGGGACACCTAGCGGGCATAGTTACGA 60
a R D R R Q R K R Q F R A L W I A R I N A -
GGTGACCGTCAGAACGGTCTGTCCTACAGCCGTCTGATCGCCGGCCTGAAGAAGGCGACC
61
CCACTGGCAGTCTTGCCAGACAGGATGTCGGCAGACTAGCGGCCGGACTTCTTCCGCTGG 120
a G D R Q N G L S Y S R L I A G L K K A T -
ATTGAGATCGACCGCAAGGTTCTGTCCGATCTGGCAGTGAACGAAAAAGCGGCGTTCGCC
121
TAACTCTAGCTGGCGTTCCAAGACAGGCTAGACCGTCACTTGCTTTTTCGCCGCAAGCGG 180
a I E I D R K V L S D L A V N E K A A F A -
SmaI
GCGATTGTCGAGAAGGCTAAAGCCGTCCTGGCTTGATCCCTTGATAGTCCCGGGCGTTGC
181
CGCTAACAGCTCTTCCGATTTCGGCAGGACCGAACTAGGGAACTATCAGGGCCCGCAACG 240
a A I V E K A K A V L A *
CCGGTGCCGAGCGTCACCGATAGGGGAAGAGCCTAGTGCCTTCCCCTATTTCGTATCTG
241
GGCCACGGCTCGCAGTGGCTATCCCCTTCTCGGATCACGAGAAGGGGATAAAGCATAGAC 300
WJP5
AGAAGGGGCTTTTGCAAAACCGACCCTTGCGTTTCTGGCGGGCCGCCTGCCGAAGCGGGA
301
TCTTCCCCGAAAACGTTTTGGCTGGGAACGCAAAGACCGCCCGGCGGACGGCTTCGCCCT 360
rbs
TCGAATCGGGCGTCAAGATAGTTCTGCAAGACCCTCTGGAGGTTGCACATGGAAAACCTG
361
AGCTTAGCCCGCAGTTCTATCAAGACGTTCTGGGAGACCTCCAACGTGTACCTTTTGGAC 420
pheS
a M E N L -
WJP4
GATGCACTGGTCGCGCAAGCGCTTGAGGCCGTGCAACATAGCGAAGACGTCAATGCCCTG
421
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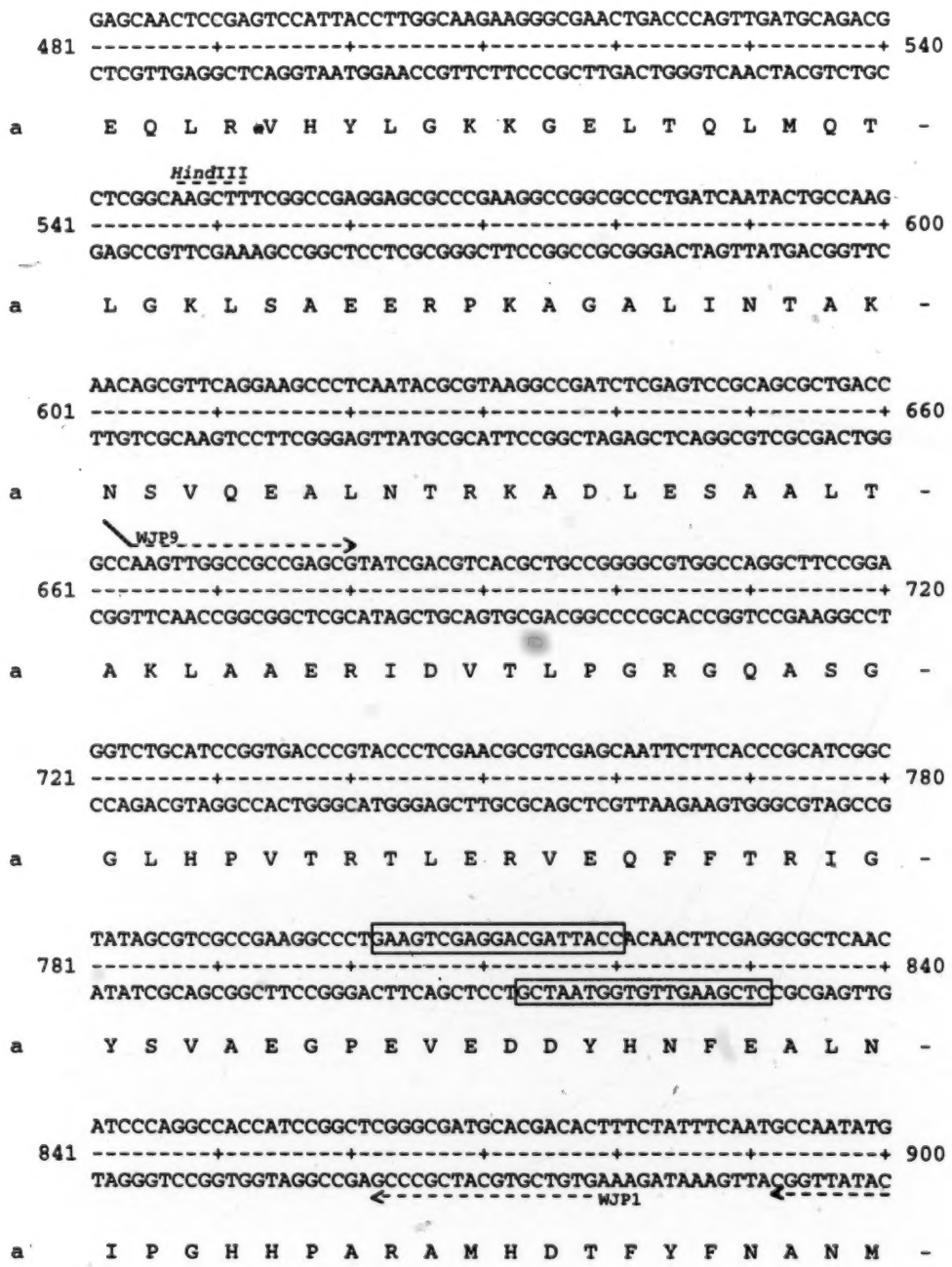
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a E Q L R V H Y L G K K G E L T Q L M Q T -
HindIII
CTCGGCAAGCTTTCGGCCGAGGAGCGCCCGAAGGCCGGCGCCCTGATCAATACTGCCAAG
541 600
GAGCCGTTCGAAAGCCGGCTCCTCGCGGGCTTCCGGCCGCGGGACTAGTTATGACGGTTC
a L G K L S A E E R P K A G A L I N T A K -
AACAGCGTTCAGGAAGCCCTCAATACGCGTAAGGCCGATCTCGAGTCCGCAGCGCTGACC
601 660
TTGTCGCAAGTCCTTCGGGAGTTATGCGCATTCCGGCTAGAGCTCAGGCGTCGCGACTGG
a N S V Q E A L N T R K A D L E S A A L T -
WJP9
GCCAAGTTGGCCGCCGAGCGTATCGACGTCACGCTGCCGGGGCGTGGCCAGGCTTCCGGA
661 720
CGGTTCAACCGGCGGCTCGCATAGCTGCAGTGCGACGGCCCCGCACCGGTCCGAAGGCCT
a A K L A A E R I D V T L P G R G Q A S G -
GGTCTGCATCCGGTGACCCGTACCCTCGAACGCGTCGAGCAATTCTTCACCCGCATCGGC
721 780
CCAGACGTAGGCCACTGGGCATGGGAGCTTGCGCAGCTCGTTAAGAAGTGGGCGTAGCCG
a G L H P V T R T L E R V E Q F F T R I G -
TATAGCGTCGCCGAAGGCCCTGAAGTCGAGGACGATTACCACAACTTCGAGGCGCTCAAC
781 840
ATATCGCAGCGGCTTCCGGGACTTCAGCTCCTGCTAATGGTGTTGAAGCTCCGCGAGTTG
a Y S V A E G P E V E D D Y H N F E A L N -
ATCCCAGGCCACCATCCGGCTCGGGCGATGCACGACACTTTCTATTTCAATGCCAATATG
841 900
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WJP1
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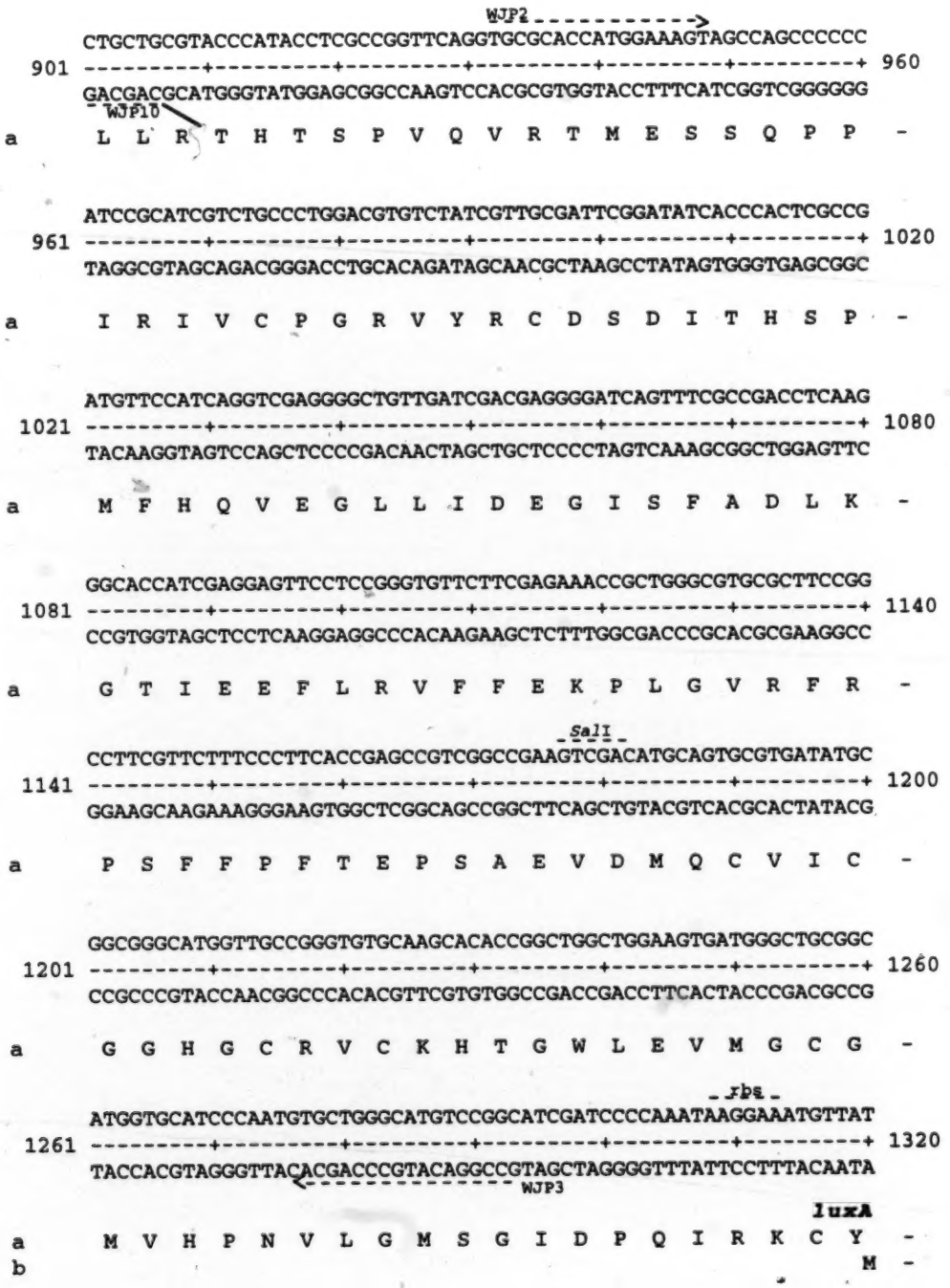
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901 960
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WJP10
a L L R T H T S P V Q V R T M E S S Q P P -
ATCCGCATCGTCTGCCCTGGACGTGTCTATCGTTGCGATTCGGATATCACCCACTCGCCG
961 1020
TAGGCGTAGCAGACGGGACCTGCACAGATAGCAACGCTAAGCCTATAGTGGGTGAGCGGC
a I R I V C P G R V Y R C D S D I T H S P -
ATGTTCCATCAGGTCGAGGGGCTGTTGATCGACGAGGGGATCAGTTTCGCCGACCTCAAG
1021 1080
TACAAGGTAGTCCAGCTCCCCGACAACTAGCTGCTCCCCTAGTCAAAGCGGCTGGAGTTC
a M F H Q V E G L L I D E G I S F A D L K -
GGCACCATCGAGGAGTTCCTCCGGGTGTTCTTCGAGAAACCGCTGGGCGTGCGCTTCCGG
1081 1140
CCGTGGTAGCTCCTCAAGGAGGCCCACAAGAAGCTCTTTGGCGACCCGCACGCGAAGGCC
a G T I E E F L R V F F E K P L G V R F R -
SalI
CCTTCGTTCTTTCCCTTCACCGAGCCGTCGGCCGAAGTCGACATGCAGTGCGTGATATGC
1141 1200
GGAAGCAAGAAAGGGAAGTGGCTCGGCAGCCGGCTTCAGCTGTACGTCACGCACTATACG
a P S F F P F T E P S A E V D M Q C V I C -
GGCGGGCATGGTTGCCGGGTGTGCAAGCACACCGGCTGGCTGGAAGTGATGGGCTGCGGC
1201 1260
CCGCCCGTACCAACGGCCCACACGTTCGTGTGGCCGACCGACCTTCACTACCCGACGCCG
a G G H G C R V C K H T G W L E V M G C G -
rbs
ATGGTGCATCCCAATGTGCTGGGCATGTCCGGCATCGATCCCCAAATAAGGAAATGTTAT
1261 1320
TACCACGTAGGGTTACACGACCCGTACAGGCCGTAGCTAGGGGTTTATTCCTTTACAATA
WJP3
luxA
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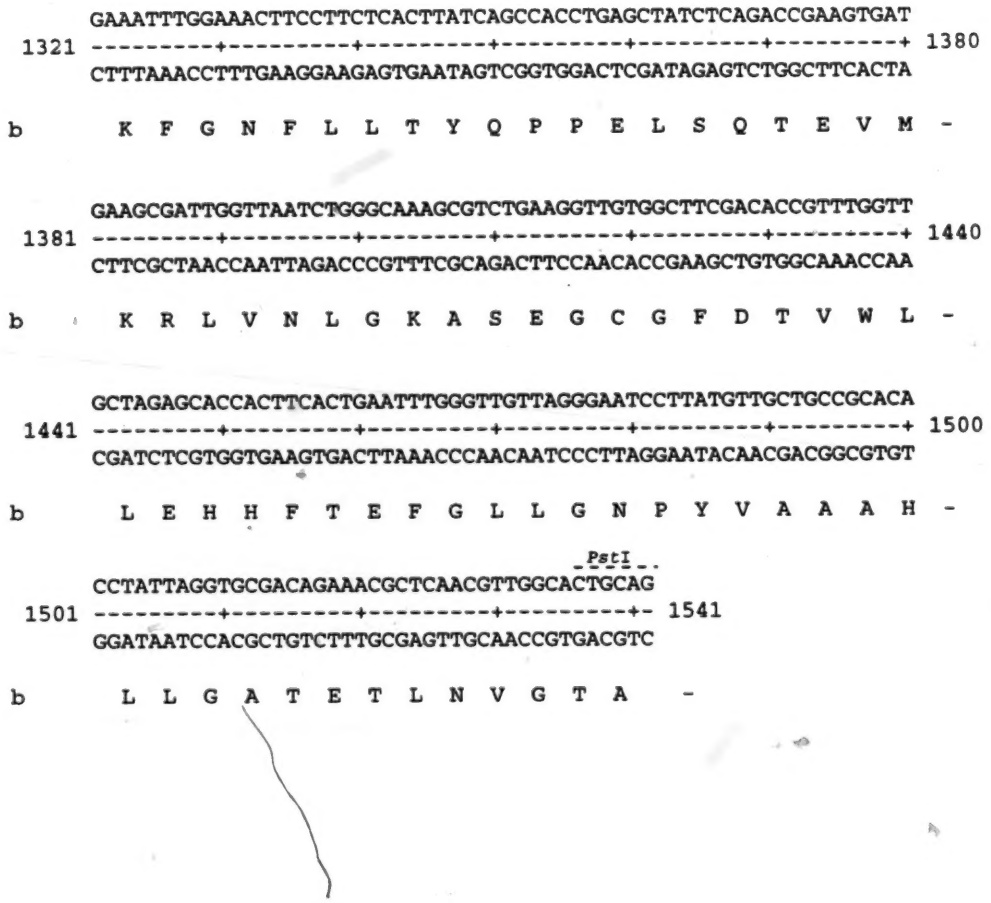
b M -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CTTTAAACCTTTGAAGGAAGAGTGAATAGTCGGTGGACTCGATAGAGTCTGGCTTCACTA
b K F G N F L L T Y Q P P E L S Q T E V M -
GAAGCGATTGGTTAATCTGGGCAAAGCGTCTGAAGGTTGTGGCTTCGACACCGTTTGGTT
1381 ---------+---------+---------+---------+---------+---------+ 1440
CTTCGCTAACCAATTAGACCCGTTTCGCAGACTTCCAACACCGAAGCTGTGGCAAACCAA
b K R L V N L G K A S E G C G F D T V W L -
GCTAGAGCACCACTTCACTGAATTTGGGTTGTTAGGGAATCCTTATGTTGCTGCCGCACA
1441 ---------+---------+---------+---------+---------+---------+ 1500
CGATCTCGTGGTGAAGTGACTTAAACCCAACAATCCCTTAGGAATACAACGACGGCGTGT
b L E H H F T E F G L L G N P Y V A A A H -
PstI
CCTATTAGGTGCGACAGAAACGCTCAACGTTGGCACTGCAG
1501 ---------+---------+---------+---------+- 1541
GGATAATCCACGCTGTCTTTGCGAGTTGCAACCGTGACGTC
b L L G A T E T L N V G T A -

Fig. 3-6 Sequence of 1.541 kb *A. vinelandii* UA22 DNA. The deduced amino acid sequence of the three open reading frames are shown, with the names of the genes written in bold. The position of synthetic oligonucleotides used in the study are shown by dashed arrows. The oligonucleotides with a non-homologous end are followed by a solid bar (WJP9 & WJP10). The putative Fur-recognition sequences are enclosed in boxes. Potential ribosome binding sites are shown with dashed lines and inverted repeats are indicated with solid converging arrows. Restriction enzyme sites of interest are also noted. The two frames are represented by a (+1) and b (+2).

```
A.v.1  RDRRQRKRQFRALWIARINAGDRQNGLSYSRLIAGLKKATIEIDRKVLSDLAVNEKAAFA  180
       RDRRQRKRQFR LWIARINA  RQNG+SYS+ I GLKKA++EIDRK+L+D+AV +K AF
E.c.48 RDRRQRKRQFRQLWIARINAAARQNGISYSKFINGLKKASVEIDRKILADIAVFDKVAFT  107

A.v.181  AIVEKAKAVLA 213
         A+VEKAKA LA
E.c.108  ALVEKAKAALA 118
```

Fig. 3-7 Comparison of the amino acid sequence encoded by a portion of UA22 DNA (A.v.) with the *rplT* gene of *E. coli* (E.c.). Identities are shown by the single letter code in the middle row, similarities by + sign, and no similarities by clear space. Identities = 53/71 (74%), Identities and Similarities = 62/71 (87%), Frame = +1.

```
A.v.409 MENLDALVAQALEAVQHSEDVNALEQLRVHYLGKKGELTQLMQTLGKLSAEERPKAGALI 588
        M +L  LVA A  A+  + DV AL+ +RV YLGKKG LT  M TL +L  EERP AGA+I
E.c.1   MSHLAELVASAKAAISQASDVAALDNVRVEYLGKKGHLTLQMTTLRELPPEERPAAGAVI 60

A.v.589 NTAKNSVQEALNTRKADLESAALTAKLAAERIDVTLPGRGQASGGLHPVTRTLERVEQFF 768
        N AK  VQ+ALN RKA+LESAAL A+LAAE IDV+LPGR   +GGLHPVTRT++R+E FF
E.c.61  NEAKEQVQQALNARKAELESAALNARLAAETIDVSLPGRRIENGGLHPVTRTIDRIESFF 120

A.v.769 TRIGYSVAEGPEVEDDYHNFEALNIPGHHPARAMHDTFYFNANMLLRTHTSPVQVRTMES 948
          +G++VA GPE+EDDYHNF+ALNIPGHHPARA HDTF+F+   LLRT TS VQ+RTM++
E.c.121 GELGFTVATGPEIEDDYHNFDALNIPGHHPARADHDTFWFDTTRLLRTQTSGVQIRTMKA 180

A.v.949 SQPPIRIVCPGRVYRCDSDITHSPMFHQVEGLLIDEGISFADLKGTIEEFLRVFFEKPLG 1128
         QPPIRI+ PGRVYR D D TH+PMFHQ+EGL++D  ISF +LKGT+ +FLR FFE+ L
E.c.181 QQPPIRIIAPGRVYRNDYDQTHTPMFHQMEGLIVDTNISFTNLKGTLHDFLRNFFEEDLQ 240

A.v.1129  VRFRPSFFPFTEPSAEVDMKHTGWLEVMGCGMVHPNVLGMSGIDPQI  1308
          +RFRPS+FPFTEPSAEVD+K+  WLEV+GCGMVHPNVL   GIDP++
E.c.241   IRFRPSYFPFTEPSAEVDVKNGKWLEVLGCGMVHPNVLRNVGIDPEV  289
```

Fig. 3-8 Comparison of the amino acid sequence of the UA22 DNA (A.v.) with the *E. coli* (E.c.) *pheS* gene. Single letter amino acid code is used, the identities are shown in the middle row by their single letter codes and the similarities with the + sign and no similarity is depicted by a clear space. Identities = 171/259 (66%), Identities and Similarities = 208/259 (80%), Frame = +1

The *Azotobacter* DNA sequence also showed considerable homology with the *pheS* and/or *rplT* genes of some bacteria such as *Bacillus subtilis* (gene bank accession number X53057), *Bacillus stearothermophilus* (X16188), *Thermus aquaticus thermophilus* (Keller *et al.*, 1992), *P. syringae* (U44118), *P. aeruginosa* (U15393), *Haemophilus influenzae* (U32810), and *Saccharomyces cerevisiae* (J03965).

Scanning of the *A. vinelandii* DNA sequence upstream of the *luxA* gene revealed two putative iron-boxes. The one on the sense strand was located between positions 801 and 821 within the *pheS* gene and the other was on the opposite strand located between positions 831-814 (Fig. 3-6). These 19 bp sequences show 53% and 58% identity with the *E. coli* consensus iron-box sequence (Table 3-4). Scanning the published *E. coli pheS* sequence (Fayat *et al.*, 1983) also revealed a putative iron-box in exactly the same location (394 bp into the *pheS* gene) that was 84% identical to the *A. vinelandii* iron-box (Table 3-4).

3.2.5 Comparison of the *E. coli pheS* gene region with that of sequenced UA22 DNA

The *E. coli pheS* gene region

In *E. coli*, the *pheS* gene is flanked by 4 other genes (Fig. 3-9). The *pheS* gene itself is 0.99 kb long. Immediately upstream of *pheS* is the *rplT* gene (Fayat *et al.*, 1983), which encodes the ribosomal protein L20 and is 354 nucleotides in length. The open reading frame of the *rplT* gene is followed by an inverted repeat, which is believed to act as a Rho-independent transcription terminator. The intergenic region between *rplT* and *pheS* genes is 450 bp in length and houses the promoter sequence for the *pheS* and *pheT* genes, which are expressed as two adjacent cistrons from the same transcription unit (Plumbridge & Springer, 1980; Springer *et al.*, 1982). The *pheT* gene encodes the β-subunit of the phenylalanine t-RNA synthetase enzyme and is 2.4 kb long. There is only a 14 nucleotide intergenic region between the *pheS* and *pheT* genes. The promoter for the *pheST* operon (p3, Fig. 3-9) is located 368 nucleotides in front of the *pheS* gene and is followed by a short open reading frame coding for a short peptide of 14 amino acids containing five phenylalanine residues. It has been suggested that this short ORF plays an important role in regulating the expression of the two downstream genes via an attenuation mechanism (Fayat *et al.*, 1983). It also has been suggested that at least 30% of the *pheST* transcript arises from the promoter upstream of the *rplT* gene (p2, Fig. 3-9).

	Sequence	Identity with the consensus
E. coli consensus iron-box	**GATAATGATAATCATTATC**	19/19
A. vinelandii putative iron-box 1	**GA**AGTCG**A**GGACG**ATTA**C**C**	10/19
A. vinelandii putative iron-box 2	**GA**GC**T**TC**AA**C**A**C**CATTA**GC**	11/19
Possible iron-box in *E. coli pheS*	**GAAATCGAAGACGATTATC**	12/19

Table 3-4 Comparison of the putative *A. vinelandii* UA22 Iron-boxes with that of the *E. coli* consensus iron-box and a possible iron-box within the *E. coli pheS* gene. The bases that share identity with the consensus are shown in bold. The bases, which show identity between *A. vinelandii* and *E. coli pheS* putative iron-boxes are underlined.

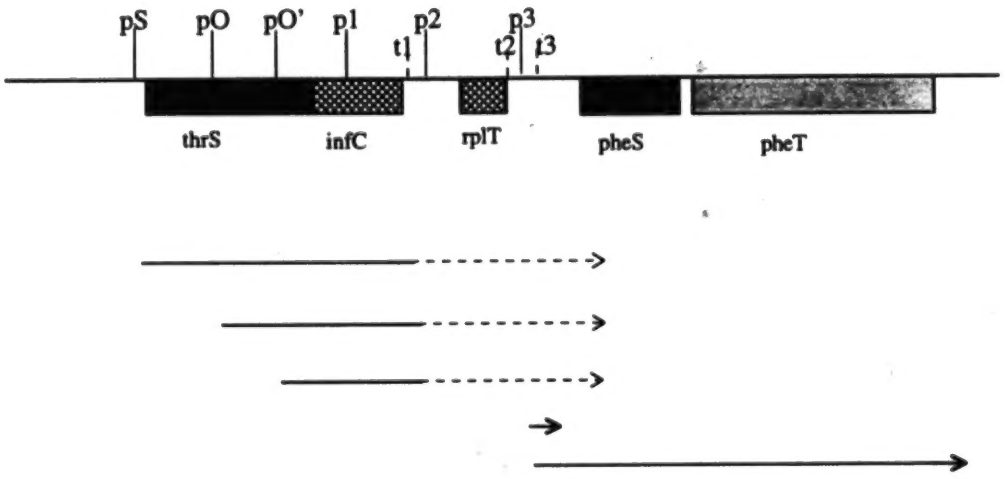


Fig. 3-9 Gene organization in the *pheS* operon of *E. coli*. The known promoters (pS,pO,pO', p1, p2, p3), terminators (t1, t2, t3), and transcriptional units (horizontal lines) in the operon, as determined by S1 mapping. Dotted lines indicate probable transcripts and the arrows indicate the direction of transcription. (Reproduced from Manago,1987)

Analysis of the UA22 DNA sequence

A few interesting differences were observed when the *A. vinelandii* UA22 DNA sequence was compared with that of the *E. coli pheS* operon. Firstly, the intergenic region between *rplT* and the *pheS* genes is only 192 bp long. Secondly, there is no ORF in this region (Fig. 3-5 and 3.6), suggesting that there is no apparent attenuator sequence present in this intergenic region.

3.3 Promoter Probe Studies

3.3.1 Subcloning of UA22 DNA in the promoter probe vector pQF50

To narrow down the region containing the promoter activity in the UA22 DNA, promoter probe studies were done. Typically, iron-box operator sequences are present in the promoter region of the iron-regulated genes. The 635 bp *Sal*I-*Hin*dIII fragment of pMVW98, which contained the putative iron-box region (boxes 1 and 2) and the flanking *pheS* sequence (Fig. 3-4 and 3.6), was subcloned into the promoter probe vector pQF50. However, there were no suitable restriction enzyme sites in the multiple cloning site of pQF50 for the insertion of this fragment to have the iron-box in the correct orientation with *lacZ* (Fig. 3-10). Therefore, the fragment was first ligated into the vector pIJ2925, digested with the same restriction enzyme sites. The ligation mixture was used to transform *E. coli* DH5α cells. The resulting clone obtained was named pM110 and was digested with *Bgl*II and *Sal*I enzymes to excise a 647 bp fragment containing the 635 bp *Sal*I-*Hin*dIII fragment. The 647 bp *Bgl*II-*Sal*I fragment was subcloned into *Bgl*II-*Sal*I sites of pQF50 (Fig. 3-10) and the ligation mixture was use to transform *E. coli* JM106 strain. The resulting clone, pM112, formed blue colonies on LB plates containing X-gal [the identity of the clone was verified by digesting pM112 with *Bgl*II-*Sal*I and analyzing the restriction enzymes digestion profile on an agarose gel]. This indicated that there was a functional promoter driving *lacZ* expression present within the 635 bp *Sal*I-*Hin*dIII fragment of the *pheS* gene of *A. vinelandii*.

To test for promoter activity in the *rplT-pheS* intergenic region upstream of the *A. vinelandii pheS* gene, the 324 bp *Sma*I-*Hind*III fragment of pMVW98 spanning this region and the start of *pheS* (Fig. 3-4 and 3.6) was also cloned into pQF50. Any promoter activity in this fragment would have driven the expression of β-galactosidase in the vector, since the fragment was in the correct orientation with the *lacZ* gene. This

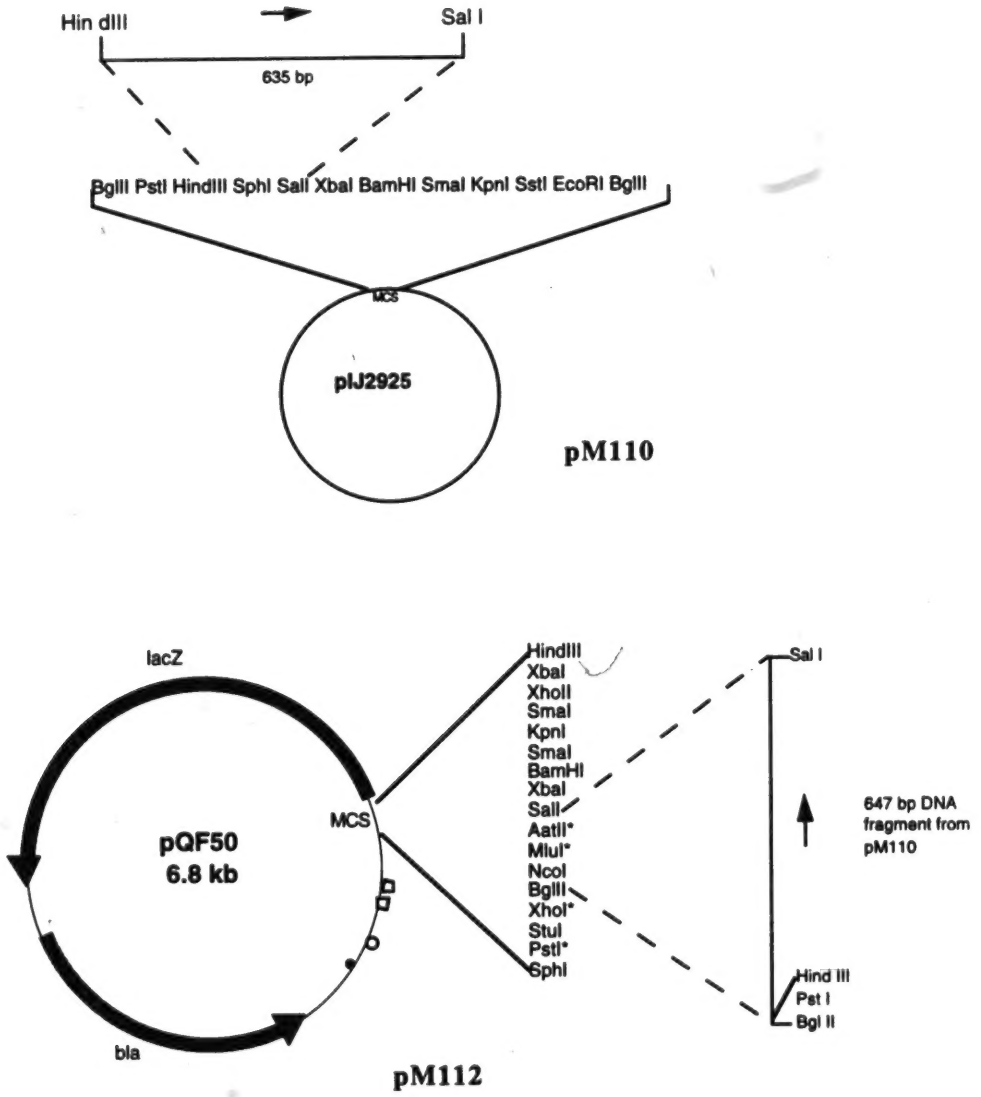


Fig. 3-10 Subcloning of 635 bp UA22 DNA fragment into the promoter probe vector pQF50. UA22 DNA fragment is first cloned into *Hin*dIII-*Sal*I sites of pIJ2925 to get a clone pM110. Thereafter 647 bp *Sal*I-*Bgl*II fragment from pM110 is excised and cloned into the same sites in pQF50. The latter clone is called pM112. • is the ori 1600, o is ori pMB1, ▭ are the two transcriptional terminators. Restriction enzyme sites of interest are noted. ⟶ denotes the direction of the promoter activity in the UA22 DNA.

construct (pM114), however, formed white colonies when plated on LB medium coated with X-gal, indicating that there is no promoter activity in the *rplT-pheS* intergenic region upstream of the *A. vinelandii pheS* gene.

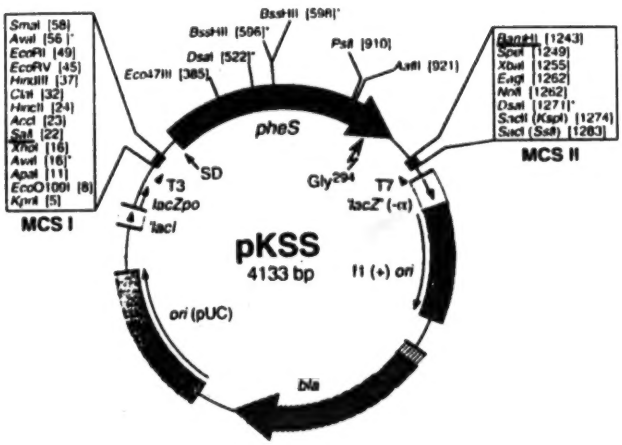
3.3.2 Subcloning of the *E. coli pheS* fragment in pQF50

Since the *A. vinelandii pheS* gene shares such a high homology with the *pheS* gene of *E. coli*, an attempt was made to check whether the latter also has promoter activity within its *pheS* gene. To do the experiment, a copy of *E. coli pheS* gene was required which lacked the normal promoter found in the *rplT-pheS* intergenic region (Fig. 3-9). One source of such a fragment was the cloning vector pKSS (kindly provided by Dr Peter Kast), which had a promoterless *pheS* gene positioned downstream of the *lac* promoter (Fig. 3-11) (Kast, 1994). This promoterless *pheS* gene was excised as a 1.2 kb *Bam*HI-*Sal*I fragment and ligated into the same sites of pQF50. The resulting clone pMKSS (in *E. coli* JM106) formed blue colonies on LB plates coated with X-gal, demonstrating that some promoter activity exists within the *E. coli pheS* gene as well.

3.3.3 β-Galactosidase assays

E. coli JM106 (pM112) was grown in liquid iron-rich (LB supplemented with 50 μM Ferric citrate) and iron-restricted (LB supplemented with 50 mg/ml EDDHA) medium and β-galactosidase activity was measured after 2 h and every hour thereafter. *E. coli* JM106 (pQF50) was used as a negative control. The results confirm that there is some promoter activity in the insert of the subclone pM112 which was derived from the *pheS* gene of *A. vinelandii* UA22 and that β-galactosidase activity is up-regulated at least 2-fold by Fe-limitation (on comparing data obtained from the cells growing in exponential phase, 2-4 hrs, Fig. 3-12).

Similarly, *E. coli* JM106 (pMKSS) was grown in iron-rich and iron-restricted medium and β-galactosidase activity was measured as above. The results (Fig. 3-13) indicate that there is some iron-regulated promoter activity within the *E. coli pheS* as well. The native RNA polymerase appeared to recognize this internal promoter better than that present in the heterologous UA22 DNA as can be seen by the increased β-galactosidase levels in Fig. 3-13 as compared to Fig. 3-12.



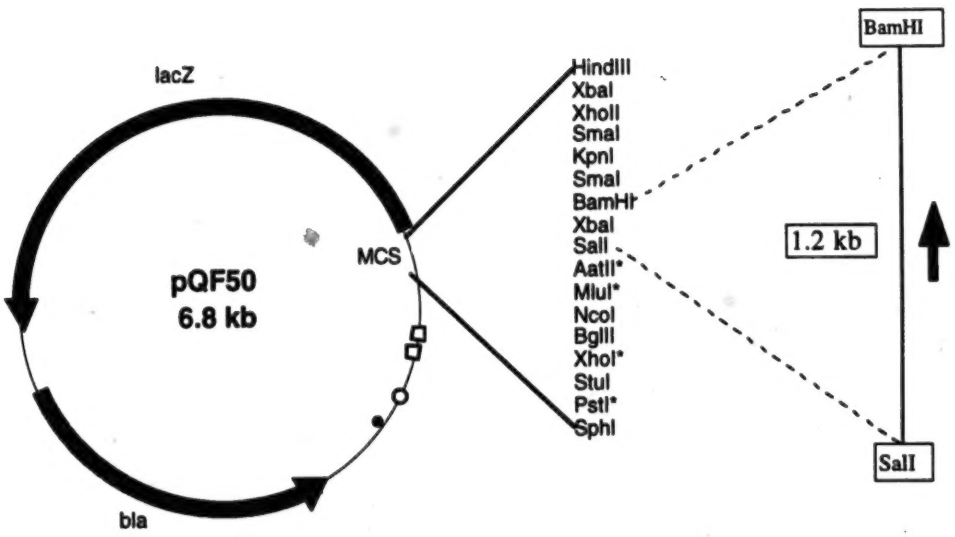


Fig. 3-11 Subcloning of a DNA fragment containing the *pheS* gene of *E. coli* in a promoter probe vector pQF50. The *E. coli pheS* gene (without its promoter region) was excised as a 1.2 kb *Bam*HI-*Sal*I fragment from the vector pKSS and ligated into the same sites in pQF50. The resulting plasmid pMKSS gave blue colonies on a plate coated with X-gal. (Figure of pKSS was reproduced from Kast, 1994.)

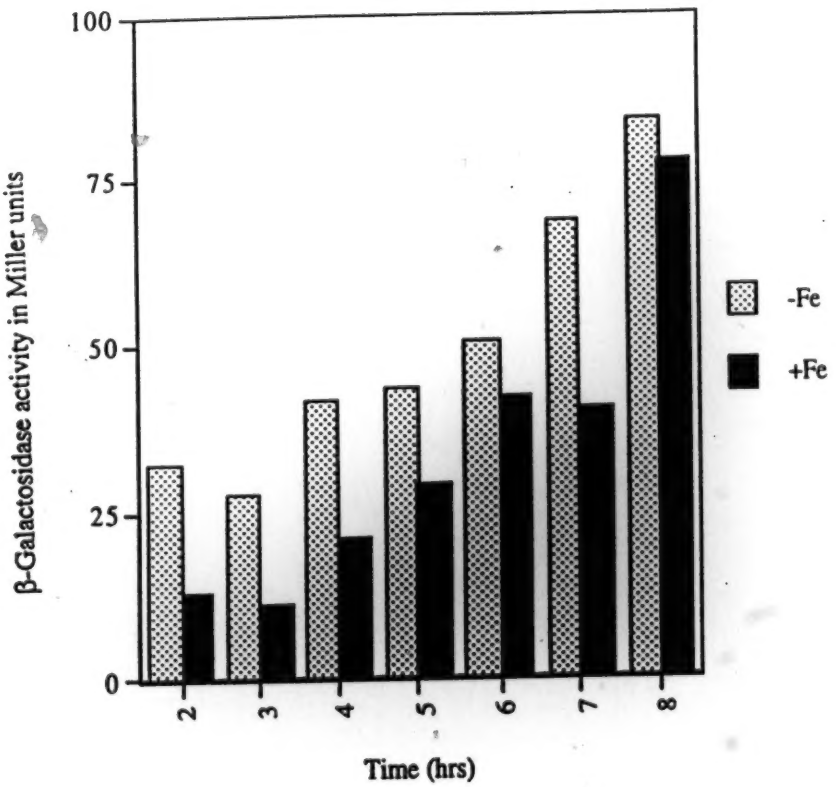


Fig. 3-12 Effect of low (-Fe) and high (+Fe) iron culture conditions on β-galactosidase activity in *E. coli* JM106 (pM112), harbouring the *A. vinelandii* UA22 DNA. Enzyme activity was measured in terms of Miller units.

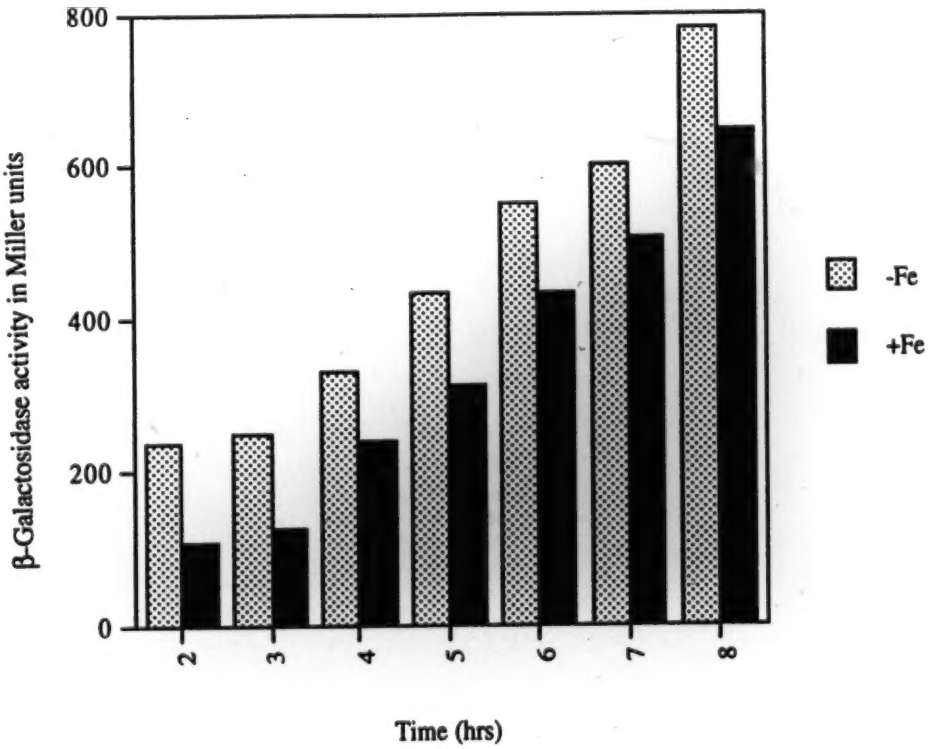


Fig. 3-13 Graph showing the comparison of β-galactosidase activities in *E. coli* strain JM106 (pMKSS), containing the *E. coli pheS* gene, grown under low iron (-Fe) and high iron (+Fe) conditions.

3.4 Southern Analysis

3.4.1 Study of the UA22 genotype

The identification of the UA22 mutation as being a disruption of the *pheS* gene by Tn*5luxAB* was an unexpected result. Such a mutation cannot conceivably be selected in *E. coli* or any other bacterium because *pheS* is an indispensable gene present in a single copy in the chromosome (Comer & Bock, 1976). Mutations that can normally be selected may affect the affinity of PheS for substrate analogues or are temperature sensitive conditional mutants (Fangman & Neidhardt, 1964; Russell & Pittard, 1971; Comer & Bock, 1976). One possible explanation for the UA22 mutation might be that the *pheS* gene is duplicated in *A. vinelandii* and exists in more than one location in the *A. vinelandii* chromosome. Another possibility is that the *pheS* gene exists in one location in the chromosome, but mutant alleles can exist heterozygously in this polyploid organism (Sadoff *et al.*, 1979 and Maldonado *et al.*, 1994).

To determine which of these alternative hypotheses is correct, genomic DNA from strains UW and UA22 was completely digested with *Eco*R1 or *Bam*H1 (these two enzymes do not cut within the 1.541 kb UA22 DNA fragment) and the location of *pheS* sequences was determined by Southern hybridization. Two probes were used: one was the internal fragment of the *A. vinelandii pheS* gene (635 bp *Hin*dIII-*Sal*I fragment of pMVW98) and the other was an internal fragment of *luxA* gene (330 bp *Pst*I fragment of pMVW97). The probes were labeled with α ^{32}P dCTP using random primer-labeling. The nylon membrane was first hybridized with the *pheS* probe, stripped, and hybridized with the *lux* probe.

According to the results obtained, an approximately 18 kb *Bam*HI or a 2.2 kb *Eco*RI fragment from strain UW hybridizes with the *pheS* probe (Fig. 3-14). In UA22, the majority of the *pheS* probe hybridizes in the same regions of the gel, but a small amount hybridizes to a larger 3.3 kb *Eco*R1 fragment or a smaller approximately 15.5 kb *Bam*H1 fragment. These fragments, which were not present in the UW DNA samples, also hybridize with the *luxA* probe (Fig. 3-14). Both restriction enzyme sites, *Eco*RI and a *Bam*HI, are present in the vector pTn*5luxAB*. An *Eco*RI site is present approximately 1.1 kb from the start of *luxA* gene, thus explaining the bigger band obtained with UA22 as compared to that of UW.

Fig. 3-14 Southern analysis of *A. vinelandii* strain UW and UA22 using a *pheS* probe (a) and a *luxA* probe (b). Lane 1, UW genomic DNA digested with *Bam*HI; lane 2, UA22 DNA digested with *Bam*HI; lane 3, UW genomic DNA digested with *Eco*RI and lane 4, UA22 genomic DNA digested with *Eco*RI. The molecular size markers are indicated in the middle.

The *Bam*HI site is located in the vector outside the *luxAB* genes and in the kanamycin cassette, which explains the fact that the band obtained with UA22 is about 2.5 kb smaller than the UW *Bam*HI digest.

The data in Fig. 3-14, shows that *pheS* exists in one location in the *A. vinelandii* chromosome, and that strain UA22 is a heterozygote containing relatively few chromosomes carrying a mutant *pheS*::Tn*5luxAB* allele, and that the majority of the *pheS* copies are wild type.

3.4.2 Effect of kanamycin selection on the number of chromosomes with the mutant copy of the *pheS* gene

The finding that *A. vinelandii* strain UA22 is a heterozygote is not very surprising as there are several reports in the literature concerning the problem of heterozygous mutants of *Azotobacter* because of the polyploid nature of the bacterium (Contreras & Casadesus, 1987). If a mutant is a heterozygote, it can become homozygous or at least the number of the copies of mutant chromosomes can increase if the selection pressure is increased (Terzaghi, 1980). To explore the possibility of such an event happening in *A. vinelandii* UA22, the strain was grown in varying concentrations of kanamycin (0, 10, and 50 μg/ml) and the DNA was extracted and subjected to Southern analysis. The nylon membrane was probed with the same two probes as used in the Section 3.4.1. The results show that the dominance of the fragments that hybridized with *pheS* and *luxA* do not increase when the strain UA22 is grown in the presence of increasing amounts of kanamycin (Fig. 3-15). This does not contradict the fact that *pheS* is an essential gene and that a certain number of normal *pheS* copies have to be maintained in the cell to keep its metabolism functioning properly. The mutant chromosome was not amplified, even if the selection pressure was increased five-fold (Fig. 3-15).

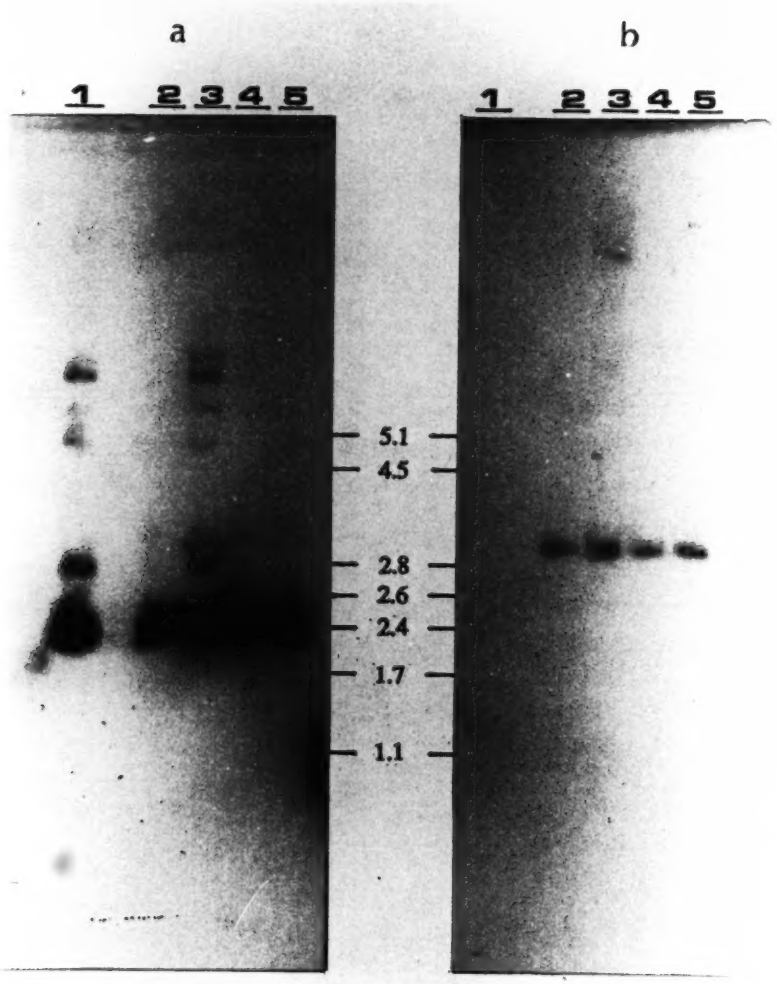


Fig. 3-15 Effect of increasing kanamycin concentrations on the mutant copy of *pheS*. Genomic DNA of *A. vinelandii* strains UW (lane1) and UA22 (lanes 2 to 5) were digested with *Eco*RI and probed with a *pheS* (a) and a *luxA* probe (b). Lane 1and 3 no kanamycin; lanes 2 and 4, 10 µg/ml kanamycin; and lane 5, 50 µg/ml kanamycin.

3.5 Gel Retardation Assays

3.5.1 Interaction of *E. coli* Fur protein with the UA22 DNA

The Fur protein is a repressor, which in the presence of a suitable corepressor (Fe^{+2} or Mn^{+2}) binds to the operator sequences of iron-regulated genes. The interaction of DNA-binding proteins with specific functional sites on linear DNA fragments can be studied by gel electrophoresis: the DNA-protein complex moves with a slower mobility than free DNA. Thus, a gel-retardation assay is a simple test to check for the presence of protein-DNA interaction.

To confirm the iron-regulation of the cloned *pheS* gene of *A. vinelandii*, considering the fact that the *A. vinelandii* iron-box is 53% identical to the *E. coli* consensus iron-box, a gel-retardation experiment was performed using the *E. coli* Fur protein. The target DNA was a 647 bp *Pst*I-*Sal*I fragment of pM110 (Fig. 3-10) containing the *A. vinelandii* UA22 *pheS* gene.

The mobility of the 647 bp *Pst*I-*Sal*I target DNA was tested with increasing concentrations of the *E. coli* Fur protein. The mobility of the 0.12 nM UA22 DNA was found to be retarded by ≈600 nM Fur protein (Fig. 3-16A). When the corepressor Mn^{2+} (Wee *et al.*, 1988 and de Lorenzo *et al.*, 1988) was excluded from the assay, no shift in DNA mobility was observed (Fig. 3-16B).

Also, no retardation was observed of DNA fragments that contained other promoters which were not iron-regulated. For example, the *lac* promoter of pUC119 was not retarded by up to 2 μM Fur (Fig. 3-17). Under the same assay conditions, the *E. coli* Fur protein retarded the mobility of a known Fur-binding aerobactin (*iuc*) operator DNA (Fig. 3-18).

To check whether the putative iron-box present in the *E. coli pheS* would bind to the native Fur protein, vector pKSS was used as the target DNA. The vector pKSS was digested with *Pst*I and *Sal*I to generate two fragments: a larger fragment of 3.2 kbp and a smaller 0.9 kbp fragment. The small fragment contained the iron-box sequence of the *E. coli pheS* gene. The retardation of this fragment was weak at ≈800 nM Fur and only very faint bands of Fur: DNA could be seen (Fig. 3-19).

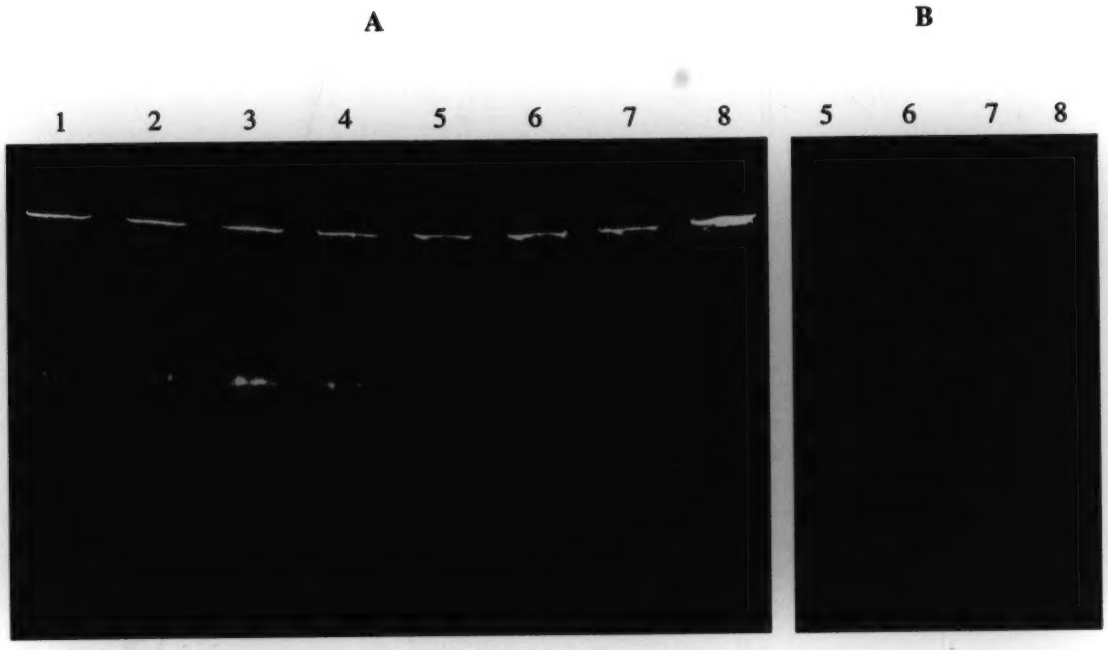


Fig. 3-16 (A) Binding of *E. coli* Fur protein to a 647 bp *Pst*I-*Sal*I fragment of pM110 harbouring a portion of *A. vinelandii* UA22 *pheS* gene. About 0.12 nM target DNA was incubated with various concentrations of Fur protein. Concentrations of the Fur protein were (nM); lane 1, 0; lane 2, 100; lane 3, 200 ; lane 4, 400 ; lane 5, 600; lane 6, 800 ; lane 7, 1000 and lane 8, 2000
(B) Lanes similar to A, but the corepressor Mn^{+2} was excluded from the assay system.

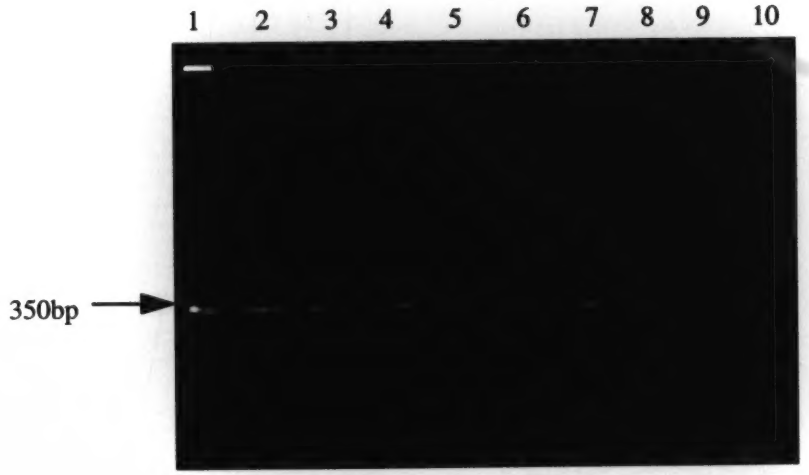


Fig. 3-17 Binding of *E. coli* Fur protein to*E. coli lac* promoter. A 350 bp *Pvu*II fragment of vector pUC119 (0.12 nM) containing the *lac* promoter region was incubated with increasing concentrations of the Fur protein. The concentrations were (nM): lane 1, 0; lane 2, 100; lane 3, 200; lane 4, 400; lane 5, 600; lane 6, 800; lane 7, 1000; lane 8, 1200; lane 9, 2000; lane 10, 2500.

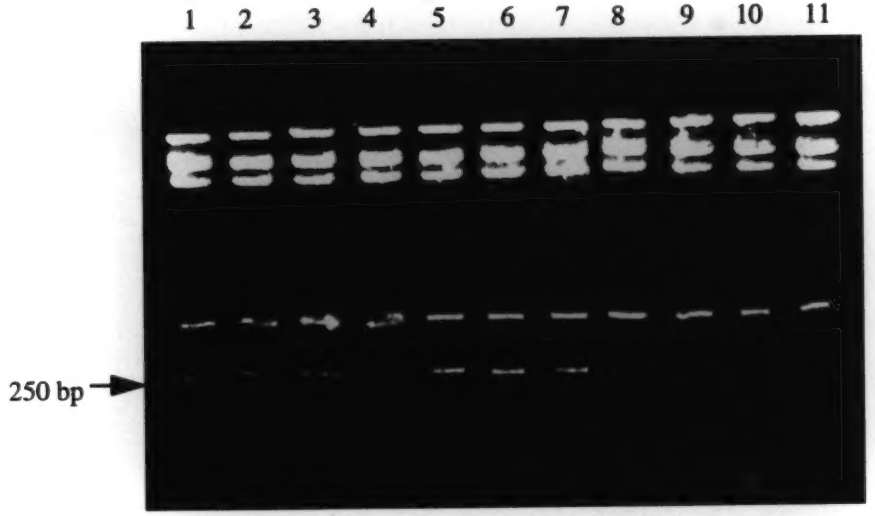


Fig. 3-18 Binding of *E. coli* Fur protein to pCON6 plasmid harbouring the *iucA* operator region of *E. coli*. The mobility of a 250bp *Eco*RI-*Pvu*II fragment was shifted with Fur binding. Target DNA was incubated with various concentrations of Fur protein (nM). Lane 1, 0; lane 2, 5; lane 3, 10; lane 4, 20; lane 5, 50; lane 6, 75; lane 7, 100; lane 8, 250; lane 9, 400; lane 10, 500; and lane 11, 1000.

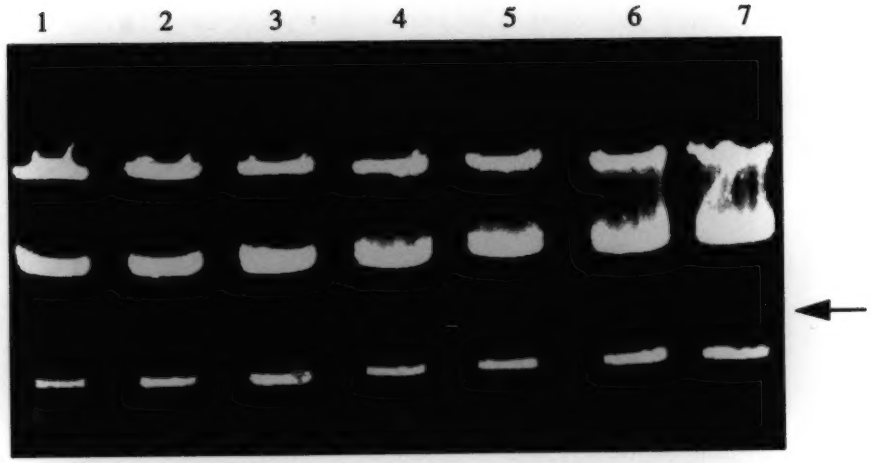


Fig. 3-19 Binding of *E. coli* Fur protein to *Pst*I-*Sal*I digested pKSS plasmid containing the *E. coli* *pheS* gene. Mobility of 0.9 kb fragment seems to be retarded slightly (shown by arrow). The concentrations of Fur protein in each lane were (nM): lane 1, 0; lane 2, 100; lane 3, 200; lane 4, 400; lane 5, 600; lane 6, 800 and lane 7, 1000.

One reason for this weak interaction could be that the target DNA size was 0.9 kbp, which is likely to be too large to give a significant gel shift under the conditions used in this assay.

3.6 Promoter Probe and Gel Retardation Studies with a PCR Product Derived from an Internal Fragment of the *A. vinelandii* UA22 *pheS* Gene

3.6.1 Promoter probe studies

To narrow down the region containing the iron-box in *A. vinelandii* UA22 DNA, a PCR fragment was synthesized using primers WJP9 and WJP10 (Fig. 3-6) with pM112 (Fig. 3-10) as the template. WJP9 was 27 bases long with an extra 10 bases at the 5' end engineered to contain an *Eco*RI site. WJP10 was also 27 bases long with an extra 10 bases attached to its 5' end engineered to contain a *Hin*dIII site. The PCR amplified fragment was 265 bp long with 245 bp of *Azotobacter* DNA and 20 bp of extra sequence containing the restriction sites. This PCR fragment was digested with *Hin*dIII and *Eco*RI and cloned into the promoter probe vector pQF50. The clone in pQF50 was called pMPCR2. It gave blue colonies on LB Amp plates containing X-gal, indicating that the PCR2 fragment has the sequence responsible for promoter activity within the *pheS* gene of *A. vinelandii*.

The promoter activity present in the plasmid pMPCR2 was quantified by the β-galactosidase assay. *E. coli* JM106 (pMPCR2) was grown overnight in LB Amp medium and then diluted 1:100 into low-iron and high-iron media. Samples were withdrawn for the assay at 1 h intervals over 8 h (Fig. 3-20). The expression of β-galactosidase was Fe-regulated as observed previously with pM112 (Fig. 3-12 and Fig. 3-20). Also, the amount of β-galactosidase activity obtained with the pMPCR2 plasmid was significantly higher than that obtained with pM112 (Fig. 3-12 and Fig. 3-20). One possible explanation for this result could be that the putative promoter sequence was positioned much closer to the *lacZ* gene in pMPCR2 than in pM112. As a negative control, *E. coli* JM106 (pQF50) was grown in the same media and the β-galactosidase activity was found to be negligible (data not shown).

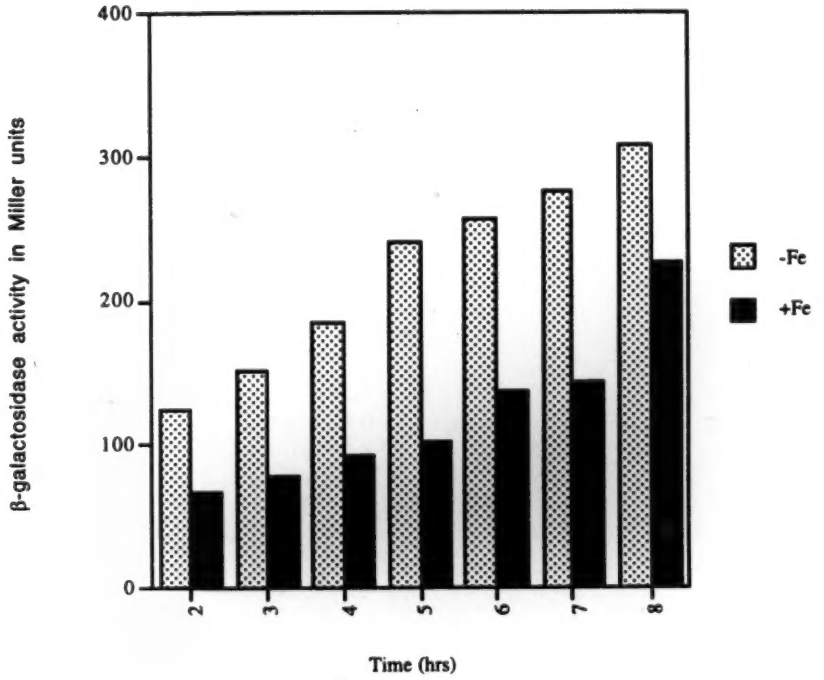


Fig. 3-20 Effect of low (-Fe) and high (+Fe) iron culture conditions on β-galactosidase activity in *E. coli* strain JM106 (pMPCR2) which contains a portion of the *A. vinelandii pheS* gene.

3.6.2 Gel retardation studies

Plasmid pMPCR2 was digested with *Hin*dIII and *Eco*RI to excise the 255 bp fragment of *A. vinelandii pheS* DNA. This was dephosphorylated and end-labeled using the enzyme polynucleotide kinase. The end-labeled *Hin*dIII-*Eco*RI fragment of pMPCR2 was diluted to ≤ 0.1 nM and used as target DNA in the reactions containing increasing amounts of Fur protein. The mobility of the target DNA was found to be retarded by ≥ 600 nM Fur protein (Fig. 3-21). When the co-repressor Mn^{+2} was excluded from the assay, no shift in mobility of the target DNA was observed (Fig. 3-22). For a negative control, the 670 bp end-labeled *Eco*RI-*Xho*I fragment of pMJH3 containing the nitrogen-regulated promoter of *A. vinelandii anfH* gene was used. The results show that the Fur protein does not recognize this fragment and there is only non-specific binding at the highest concentration of 2-4 μM Fur (Fig. 3-23 and Fig. 3-24). For a positive control, the 250 bp *Eco*RI-*Pvu*II fragment of pCON6 containing the *E. coli iucA* promoter was used. These results testify to the presence of two defined Fur-bound complexes (Fig. 3-25) as was shown by de Lorenzo *et al.* (1988). In the absence of Mn^{+2}, no band shift was observed in the range of the Fur concentrations assayed (Fig. 3-26).

These results confirm the presence of an iron-box within the *pheS* gene of *A. vinelandii* which was recognized by the *E. coli* Fur protein that bound in a corepressor-dependent manner.

3.7 Transcriptional Analysis of the *pheS* Gene of *A. vinelandii*

3.7.1 S1 nuclease protection assay

The S1 nuclease enzyme is a single-stranded endonuclease that will digest both single-stranded RNA and DNA. The principle of S1 nuclease analysis involves the following steps: (i) hybridize a 5' end-labeled DNA probe fragment to cellular RNA; (ii) S1 nuclease is then added to digest all single-stranded regions: 5' overhangs, 3' overhangs and introns, depending on the specific probe used. The double-stranded RNA-DNA hybrid is resistant to cleavage. Electrophoresis of the hybrid on a denaturing polyacrylamide gel allows a determination of the length of the remaining DNA fragment.

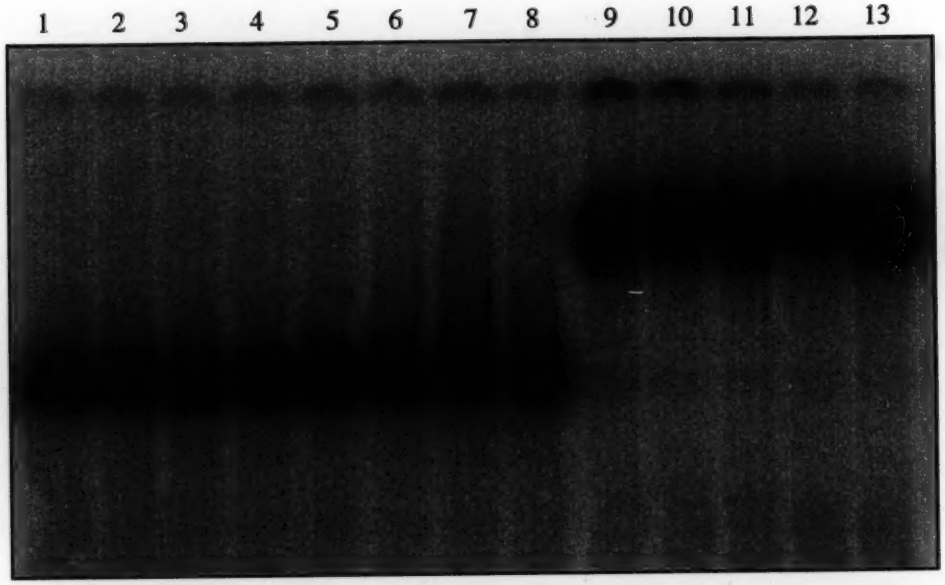


Fig. 3-21 Binding of the *E. coli* Fur protein to a 255 bp *Hin*dIII-*Eco*RI fragment of pMPCR2 which contains a portion of the *A. vinelandii* UA22 *pheS* gene. Less than 0.1 nM of end-labeled target DNA was incubated with various concentrations of Fur protein. Concentrations of protein used in each lane were (nM): lane 1, 0; 2, 25; 3, 50; 4, 100; 5, 200; 6, 300; 7, 400; 8, 500; 9, 600; 10, 800; 11, 1000; 12, 2000 and 13, 4000.

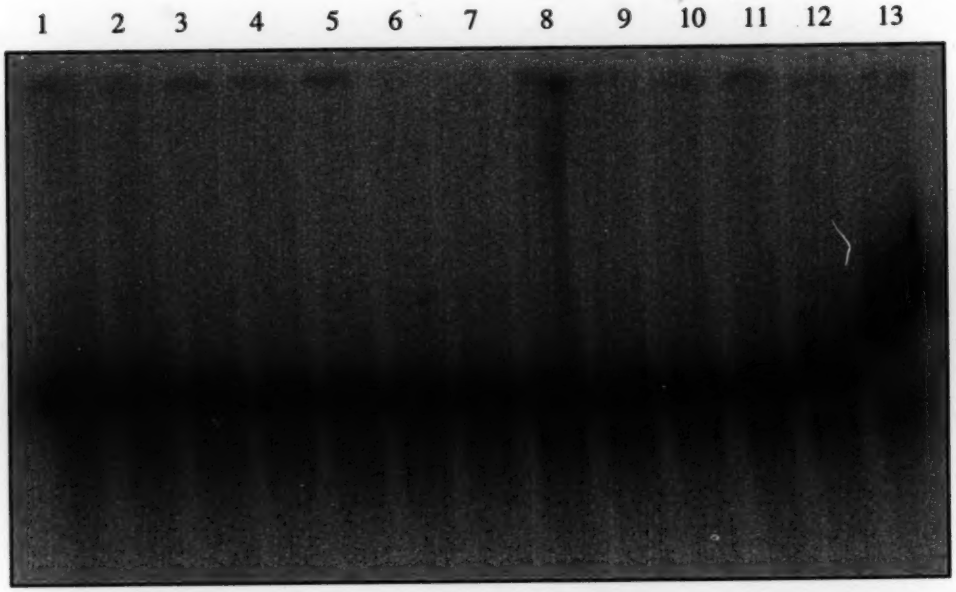


Fig. 3-22 Binding of the *E. coli* Fur protein to a 255 bp *Hin*dIII-*Eco*RI fragment of pMPCR2 containing the *A. vinelandii* UA22 *pheS* gene. The corepressor Mn^{+2} was excluded from the assay. Other conditions used were the same as described for Fig. 3-21.

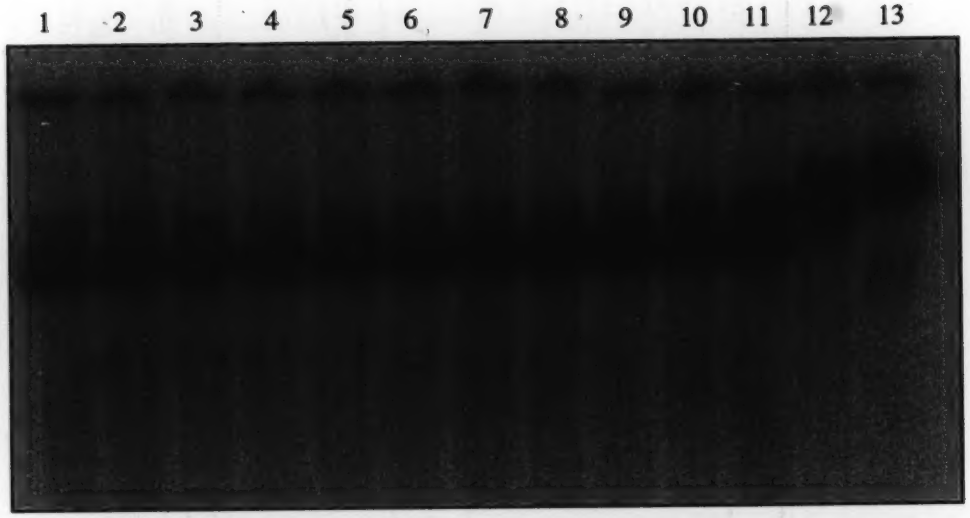


Fig. 3-23 Binding of the *E. coli* Fur protein to a 670 bp *Eco*RI-*Xho*I fragment of pMJH3, containing the nitrogen-regulated promoter region of *A. vinelandii anf*H gene. The assay conditions used were identical to those described for Fig 3-21.

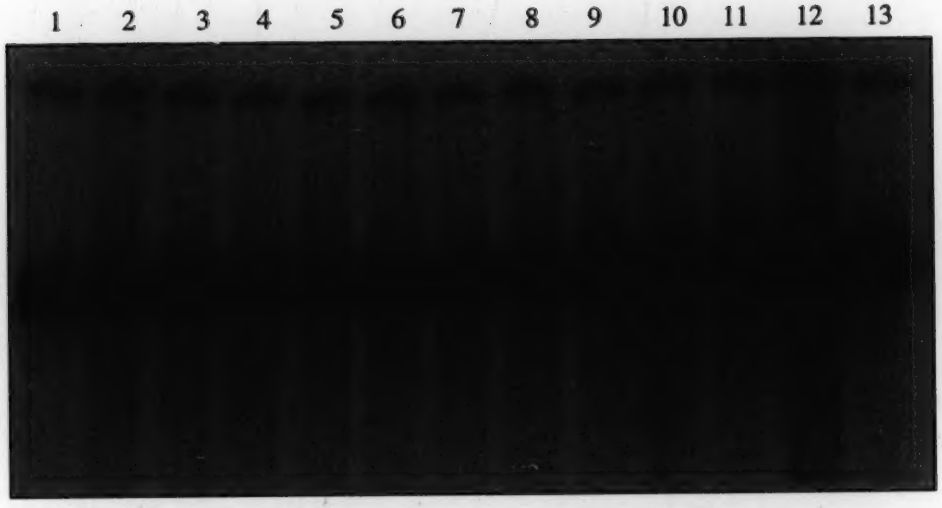


Fig. 3-24 Binding of the *E. coli* Fur protein to a 670 bp *Eco*RI-*Xho*I fragment of pMJH3 carrying the nitrogen-regulated promoter of *A. vinelandii anf*H gene. The corepressor Mn^{+2} was excluded from the assay. The Fur concentrations used were identical to those described for Fig. 3-21.

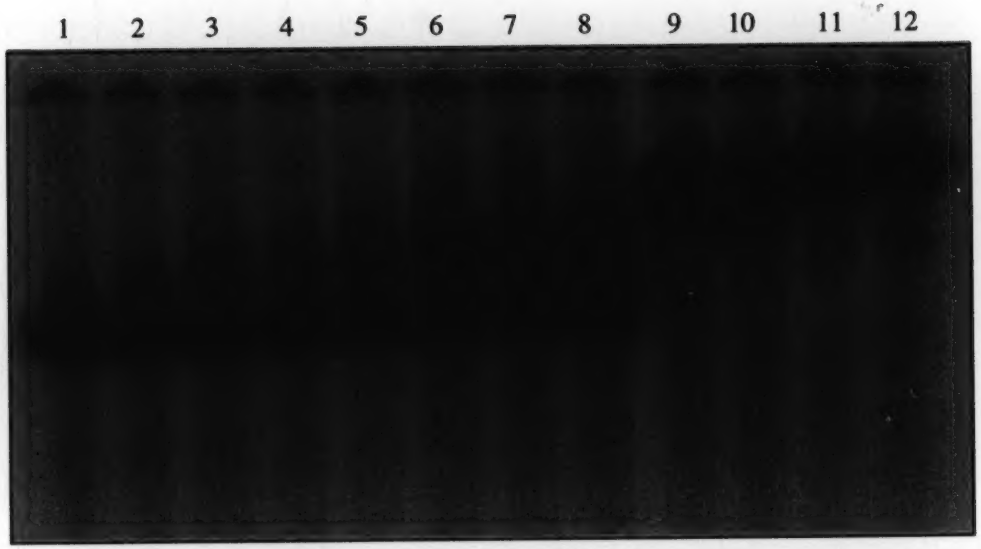


Fig. 3-25 Binding of the *E. coli* Fur protein to a 250 bp *Eco*RI-*Pvu*II fragment of plasmid pCON6 containing the *iucA* operon of *E. coli*. Less than 0.1 nM of end-labeled target DNA was incubated with various concentrations of *E. coli* Fur protein (in nM): lane 1, 0; lane 2, 25; lane 3, 50; lane 4, 100; lane 5, 200; lane 6, 300; lane 7, 400; lane 8, 500; lane 9, 600; lane 10, 800; lane 11, 1000 and lane12, 2000.

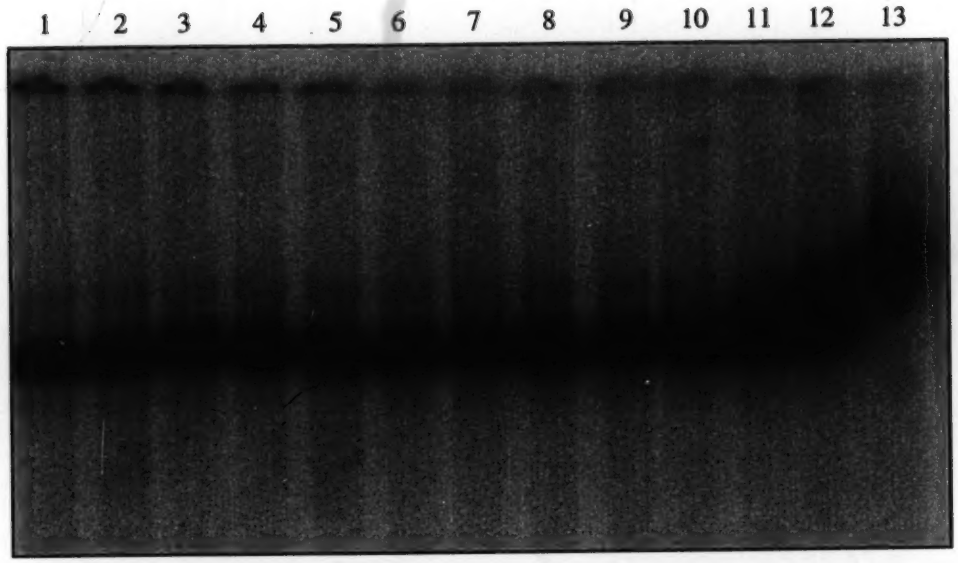


Fig. 3-26 Binding of the *E. coli* Fur protein to a 250 bp *Eco*RI-*Pvu*II fragment of pCON6 containing the *iucA* operon of *E. coli*.., Mn^{+2} the corepressor for the Fur protein was excluded from the assay system. The concentrations of Fur used in lanes 1 to 12 were identical to those described for Fig. 3-25. In lane 13, 4 μM of Fur was used.

This length equals the distance between the 5' end of the probe to the 5' end of the RNA, defining the transcriptional start site to the nucleotide (Ausubel *et al.*, 1996).

To determine whether there is a transcript start site within the *pheS* gene of *A. vinelandii in vivo*, S1 nuclease analysis was performed. For RNA isolation, cells were grown to late log-phase or early stationary phase, since in the initial experiments done with UA22, Lux activity was seen in cultures at this time. Hence, *A. vinelandii* strains UW and UA22 were grown for ≈ 20 h in low or high iron media. *E. coli* strain MV1193 (pUC119), the negative control, was also grown in low-iron for 6 h or in high-iron media for 8 h. Total cellular RNA was isolated from the cells and 50 µg of RNA from each sample was used for S1 analysis. End-labeled PCR1 (a 286 bp internal fragment of UA22 *pheS* gene, amplified by using primers WJP8 and WJP9, Fig. 3-6) was used as the probe and the S1 nuclease resistant RNA-DNA hybrid was analyzed on a 6% polyacrylamide sequencing gel. No definite conclusion could be drawn from the S1 nuclease results since the only band showing on the autoradiogram was that of the probe at all time points (data not shown).

3.7.2 Northern analysis

Northern blot analysis was done as an alternative to S1 protection studies in order to demonstrate the existence of a transcript initiating within the *pheS* gene of *A. vinelandii* UA22. RNA was isolated from *A. vinelandii* strains UW and UA22 and *E. coli* strains MV1193 (pMVW98) and MV1193 (pUC119) grown under low and high iron conditions. The membrane was probed with PCR1 and examined using the phosphoimager.

A strong transcript is visible in the lane containing the RNA sample from *E. coli* MV1193 (pMVW98) (Fig. 3-27). The intensity of the band is higher for the sample extracted from cells grown under low-iron conditions as compared to the one extracted from high-iron conditions (Fig. 3-27). However, the transcript seemed to be quite unstable and gave a smeared pattern of transcripts in both samples. No conclusion could be drawn about the transcript produced from this construct as the trancription could be driven from the *lac* promoter.

No transcript could be seen in the lanes containing RNA samples from the negative control, *E. coli* MV1193 (pUC119) (Fig. 3-27).

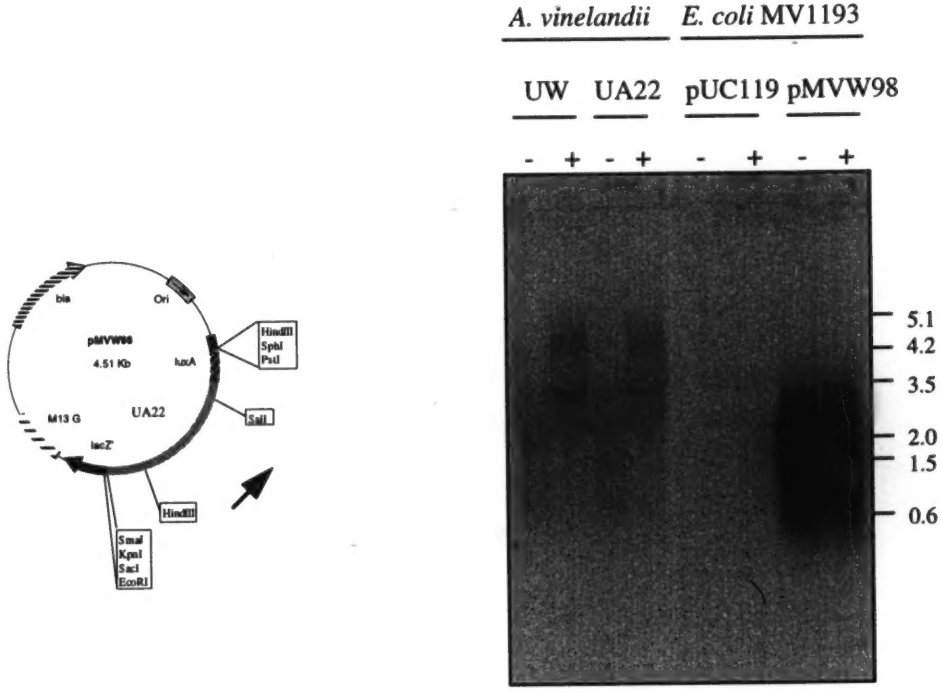


Fig. 3-27 Northern analysis of the *pheS* transcript in UW, UA22 and *E. coli* containing pUC119 or pMVW98. *A. vinelandii* strains were grown for 20 hrs in low (-) and high (+) iron conditions. *E.coli* strains were grown in low Fe for 8 hrs and in high Fe for 6 hrs. RNA was isolated from the samples and blotted onto the membrane and probed with PCR1 fragment of UA22. Schematic of pMVW98 is shown on the left with the arrow indicating the direction of promoter activity within the insert DNA with respect to the vector promoter.

Very faint bands are visible in the *A. vinelandii* RNA samples (Fig. 3-27). Since these RNA samples were taken from only one time point, there was no information on when the *pheS* transcript was maximally expressed. A time course experiment was therefore done.

3.7.3 Time course Northern analysis

For the *A. vinelandii* time course, RNA was extracted from strains UW and UA22 from 13 to 21 h at 2 h intervals. Forty μg of RNA was used in Northern analysis and the probe was an end-labeled synthetic oligonucleotide, WJP1. An oligonucleotide probe was used so that the probe was strand-specific so that there could be no non-specific hybridization with transcript arising from the opposite strand. There were no transcripts visible on the Northern blots indicating that there was no transcript arising from within the *pheS* gene of *A. vinelandii*.

3.7.4 Primer extension analysis

This technique is used for precise localization of the 5' terminus of an mRNA. A synthetic oligonucleotide primer of about 17 to 30 bases in length is labeled at its 5' end using polynucleotide kinase. Ideally, the primer should be designed such that it hybridizes to the mRNA within 50-250 bases of the candidate 5' end. The labeled primer is annealed to the mRNA (20-40 μg) under reasonably stringent conditions. After annealing, the primer is extended using the RNA-dependent DNA polymerase activity of reverse transcriptase, which elongates the primer until the template ends at the 5' end of the mRNA. The primer-extended fragments are resolved on a 6% denaturing polyacrylamide gel, using a sequencing ladder to determine the length of the primer-extended species (Ausubel *et al.*, 1996).

Primer extension analysis was used as another method to demonstrate whether a transcript is initiated from within the *pheS* gene of *A. vinelandii*. To localize the site of *in vivo* transcription initiation from within the *pheS* gene, total cellular RNA was isolated from *E. coli* JM106 (pMPCR2) and JM106 (pQF50) grown under low-iron and high-iron conditions. Total cellular RNA from *A. vinelandii* strains UW and UA22 grown under low-iron conditions was used as well. Reverse transcription of mRNA primed by a end-labeled synthetic oligonucleotide (WJP1), representing the antisense of nucleotide positions 862 to 878 in Fig. 3-6, was studied. The primer extension resulted

in only one major product (Fig. 3-28 and Fig. 3-29), albeit very weak, corresponding to a putative transcript initiation site (+1) at the T/C residue at position 826/827 in Fig. 3-30. The site was positioned near the 3' terminus of the *A. vinelandii* sequences strongly resembling the consensus *E. coli* Fur-binding site spanning nucleotide positions 802 to 820.

3.8 Identification of a Fur-like Protein in *A. vinelandii*

3.8.1 Preliminary evidence

The fact that the *A. vinelandii* IRP-*luxAB* and IRP-*lacZ* reporter constructs exhibited Fe-repressible activity in *E. coli* strongly suggested that the *E. coli* Fur repressor recognized the *A. vinelandii* iron-box. Preliminary studies done by M.Vande Woestyne did show evidence of Fur-mediated repression of Lux activity. In these studies, pMVW31 was transformed into the *E. coli* strains BN402 (Fur-positive) and BN4020 (Fur-minus), generating the strains BN40231 and BN402031, respectively. These strains were grown overnight on split Petri plates containing LB medium alone or LB containing 300 μM ferric citrate and bioluminescence was assayed by Petri plate contact printing (Vande Woestyne & Page, unpublished). Both strains showed similar bright (+++) bioluminescence in LB medium, which had become Fe-depleted during incubation. However, light production was also derepressed (+++) when the Fur-minus strain BN402031 was grown on Fe-sufficient LB medium, rather than repressed (+) as observed in the Fur-positive strain BN40231. This was the first evidence that *A. vinelandii* IRP could to be regulated by *E. coli* Fur.

3.8.2 Western blot analysis

In an effort to show that a Fur-like protein also exists in *A. vinelandii* , Western blot analysis was done. *A. vinelandii* strain UW was grown in iron-limited conditions, so that all the Fur, if present, would not be bound to the DNA. As negative and positive controls, *E. coli* strains BN4020 (Fur^-) and BN402 (Fur^+) were grown in LB medium overnight.

Cytoplasmic extracts were prepared and 20 to 100 μg of soluble protein was resolved on a 15% SDS-polyacrylamide gel. The proteins were transferred onto a nylon

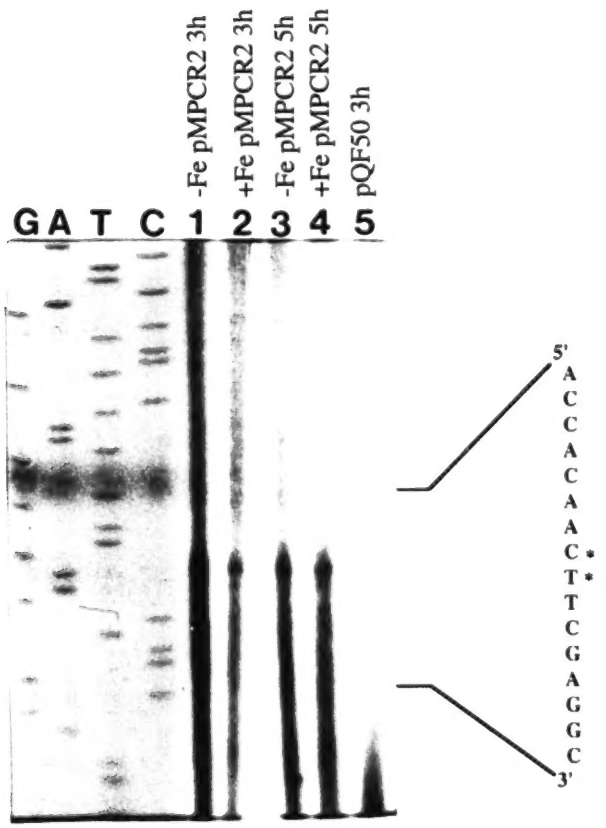


Fig. 3-28 Primer extension analysis with total RNA extracted from *E. coli* JM106 containing pMPCR2 (harbouring the internal fragment of *A. vinelandii* UA22 *pheS* gene) (lanes 1 to 4) or pQF50 (lane 5) grown in low-iron (lanes 1, 3 and 5) and high-iron (lanes 2 and 4) for 3 h (lanes 1, 2 and 5) or 5 h (lanes 3 and 4). WJP1 was used as the primer and the extended product was analyzed alongside the sequence generated using the same primer. The putative transcription start point (indicated by *) could be either C or T.

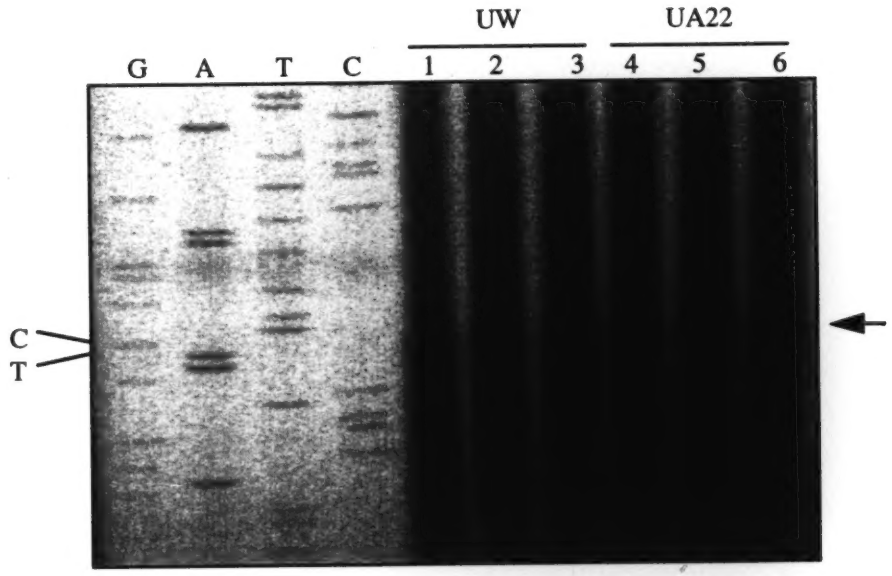


Fig. 3-29 Primer extension analysis of *A. vinelandii* UW and UA22 *pheS* gene internal promoter region. Total cellular RNAs were extracted from *A. vinelandii* UW and UA22 grown in iron-deplete conditions (1 μM ferric citrate) after 13 (lanes 1 & 4), 15 (lanes 2 & 5) and 17 (lanes 3 & 6) h. Total RNAs were hybridized with a synthetic oligonucleotide(WJP1) which is complementary to the sequence within the *pheS* gene. The extension products (◄—) were analyzed alongside the sequence, which was generated using the WJP1 as primer. The putative +1 site could be either C or T.

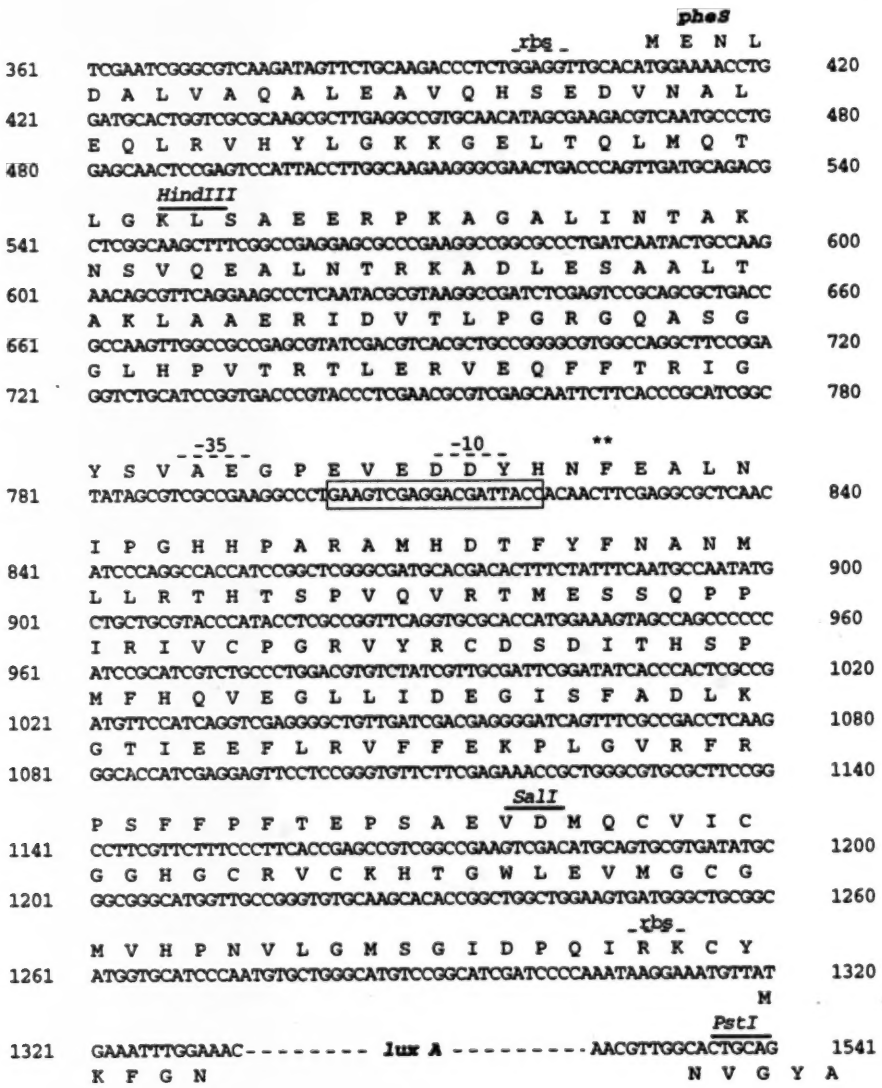

phoS
rbs
M E N L
361 TCGAATCGGGCGTCAAGATAGTTCTGCAAGACCCTCTGGAGGTTGCACATGGAAAACCTG 420
D A L V A Q A L E A V Q H S E D V N A L
421 GATGCACTGGTCGCGCAAGCGCTTGAGGCCGTGCAACATAGCGAAGACGTCAATGCCCTG 480
E Q L R V H Y L G K K G E L T Q L M Q T
480 GAGCAACTCCGAGTCCATTACCTTGGCAAGAAGGGCGAACTGACCCAGTTGATGCAGACG 540
HindIII
L G K L S A E E R P K A G A L I N T A K
541 CTCGGCAAGCTTTCGGCCGAGGAGCGCCCGAAGGCCGGCGCCCTGATCAATACTGCCAAG 600
N S V Q E A L N T R K A D L E S A A L T
601 AACAGCGTTCAGGAAGCCCTCAATACGCGTAAGGCCGATCTCGAGTCCGCAGCGCTGACC 660
A K L A A E R I D V T L P G R G Q A S G
661 GCCAAGTTGGCCGCCGAGCGTATCGACGTCACGCTGCCGGGGCGTGGCCAGGCTTCCGGA 720
G L H P V T R T L E R V E Q F F T R I G
721 GGTCTGCATCCGGTGACCCGTACCCTCGAACGCGTCGAGCAATTCTTCACCCGCATCGGC 780
-35 -10 **
Y S V A E G P E V E D D Y H N F E A L N
781 TATAGCGTCGCCGAAGGCCCTGAAGTCGAGGACGATTACCACAACTTCGAGGCGCTCAAC 840
I P G H H P A R A M H D T F Y F N A N M
841 ATCCCAGGCCACCATCCGGCTCGGGCGATGCACGACACTTTCTATTTCAATGCCAATATG 900
L L R T H T S P V Q V R T M E S S Q P P
901 CTGCTGCGTACCCATACCTCGCCGGTTCAGGTGCGCACCATGGAAAGTAGCCAGCCCCCC 960
I R I V C P G R V Y R C D S D I T H S P
961 ATCCGCATCGTCTGCCCTGGACGTGTCTATCGTTGCGATTCGGATATCACCCACTCGCCG 1020
M F H Q V E G L L I D E G I S F A D L K
1021 ATGTTCCATCAGGTCGAGGGGCTGTTGATCGACGAGGGGATCAGTTTCGCCGACCTCAAG 1080
G T I E E F L R V F F E K P L G V R F R
1081 GGCACCATCGAGGAGTTCCTCCGGGTGTTCTTCGAGAAACCGCTGGGCGTGCGCTTCCGG 1140
SalI
P S F F P F T E P S A E V D M Q C V I C
1141 CCTTCGTTCTTTCCCTTCACCGAGCCGTCGGCCGAAGTCGACATGCAGTGCGTGATATGC 1200
G G H G C R V C K H T G W L E V M G C G
1201 GGCGGGCATGGTTGCCGGGTGTGCAAGCACACCGGCTGGCTGGAAGTGATGGGCTGCGGC 1260
rbs
M V H P N V L G M S G I D P Q I R K C Y
1261 ATGGTGCATCCCAATGTGCTGGGCATGTCCGGCATCGATCCCCAAATAAGGAAATGTTAT 1320
M
PstI
1321 GAAATTTGGAAAC--------lux A----------AACGTTGGCACTGCAG 1541
K F G N N V G Y A

Fig. 3-30 A portion of UA22 DNA sequence showing the position of putative iron-box and possible transcriptional start sites (indicated by *). Sequences showing weak homology to *E. coli* -10 and -35 promoter determinants are also shown. (Refer legend of Fig. 3-6, for more details).

membrane which, after suitable washing and blocking steps, was reacted with either rabbit anti-*E. coli* Fur or rabbit anti-*P. aeruginosa* Fur as the primary antibody. After overnight incubation, the membrane was treated with the secondary antibody, anti-rabbit goat polyclonal antibody conjugated to alkaline phosphatase. The appearance of blue bands on the membrane from conversion of BCIP substrate into 5,5'-dibromo-4,4'dichloroindigo by the action of alkaline phosphatase indicated the presence of the Fur protein.

A Fur-like protein was revealed in *A. vinelandii* UW at a molecular weight of 17-18 kDa range characteristic of the *E. coli* Fur protein (Fig. 3-31). The intensity of the band was found to be much brighter when anti-Fur from *Pseudomonas* was used. There was no band visible in the lane containing *E. coli* BN4020 (Fur^-) cytoplasmic extract, but there was a band in the BN402 (Fur^+) lane at the same position as the band in the lane containing the pure Fur protein (Fig. 3-31).

3.9 Transformation of *A. vinelandii recA*$^-$ Strain VK20[3]

In an effort to study the promoter activity of the cloned UA22 DNA in a homologous background, transformation of the plasmids pM112 or pMPCR2 into a recombination-deficient background of *A. vinelandii* was required. Strain VK20 is a *recA*$^-$ deletion mutant of *A. vinelandii,* constructed by homologous recombination (Venkatesh *et al.*, 1990). However, this strain was found to be non-competent by natural means, which necessitated the development of an electroporation method to move plasmids into VK20.

3.9.1 Electroporation

Electroporation has been widely used for introducing DNA into bacterial strains that cannot be transformed or for which transformation protocols have not been established. The method uses a high voltage electrical discharge to produce temporary pores in cells through which DNA can enter (Shigekawa and Dower, 1988).

Trevors & Starodub (1990) described electroporation of a capsule positive strain of *A. vinelandii* (ATCC 12837). In their study, the highest number of transformants (2.75×10^4 transformants per µg plasmid DNA) were obtained when electroporation conditions were 1500 V/0.4 cm electrode distance, 25 µF capacitance, and 29 millisec

[3] Work on this section was done jointly with Anne Sharpe, MSc candidate, in the laboratory of W.J.Page

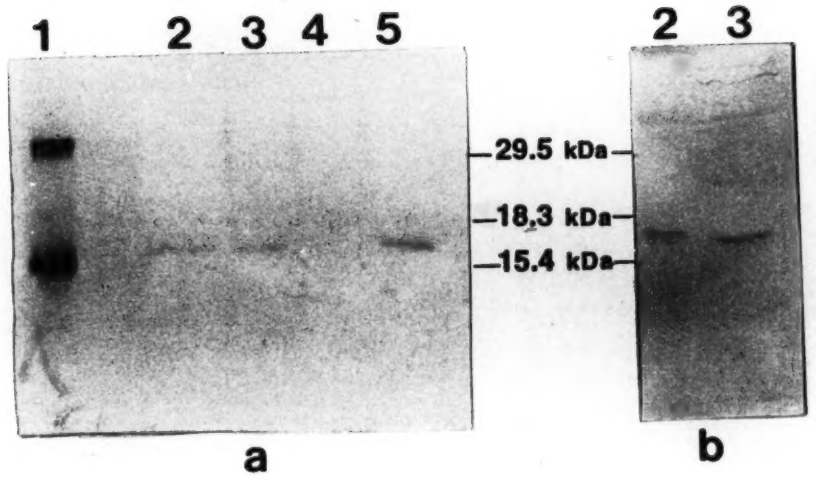


Fig. 3-31 Identification of a Fur-like protein in *A. vinelandii*. Western blot (a) with anti-*E. coli* Fur antiserum and pure Fure protein from *E. coli* (lane 1), *A. vinelandii* cytoplasmic extract (lane 2, 20 µg; lane 3, 40 µg), cytoplasmic extract of *E. coli* BN4020 (Fur^-), cytoplasmic extract of *E. coli* BN402 (Fur^+) ; (b) with anti-*P. aeruginosa* Fur antiserum and *A. vinelandii* cytoplasmic extract (lanes same as in a).

time constant. A procedure for electroporating the non-capsulate strains of *A. vinelandii* (i.e. UW and UA22) was not described.

3.9.2 Electroporation conditions

Electroporation conditions were determined using strain UW starting from standard *E. coli* electroporation conditions (Shigekawa & Dower, 1988): resistance of 200 ohms, capacitance of 25 μFaradays, and cell electrode distance of 0.2 cm. The voltage was varied, ranging from 0.75 kV to 2.5 kV. It was found that 2.5 kV setting gave the maximum number of transformants of strain UW, which was similar to the setting used for *E. coli*.

These conditions were applied to transform VK20 with a range of plasmids. The results show that VK20 is readily transformed by electroporation and the size of the plasmid does not seem to have much correlation with the frequency of transformation, except that the largest plasmid, pLAFR3, transformed at a very low frequency (Table 3-5).

3.9.3 Transformation of electrocompetent *A. vinelandii* strains UW and VK20 with linear versus circular DNA.

A study performed by Doran *et al.* (1987) demonstrated that covalently closed circular and open-circular forms of the plasmid pKT210 transformed naturally competent *A. vinelandii* cells at equal frequency, but the linearised form of the plasmid transformed 2-3 times more efficiently.

Plasmid pQF50 was used to compare the transformation of electrocompetent UW or VK20 with linear or circular DNA. Linear pQF50 transformed both the strains better than the circular form of the plasmid (Table 3-6). Another interesting point was that strain VK20 had a higher frequency of transformants compared to strain UW.

3.9.4 Studies with VK20 (pM112) and VK20 (pMPCR2)

Untransformed VK20 grew better in iron-sufficient medium (BBGN) than in iron-depleted medium (OFeBBGN), but grew slowly in all media compared to the wild-type UW or any of the other *A. vinelandii* strains used in this study. Strain VK20

Plasmids used	Reference	Selectable marker	Size of the plasmids (kilobases)	Frequency of transformants per viable cell
pACYC184	Chang & Cohen , 1978	Chloramphenicol	4.2	7.2×10^{-1}
pQF50	Farinha, 1990	Ampicillin	6.8	3.6×10^{-4}
pMPCR2	This study	Ampicillin	7.1	8.7×10^{-4}
pKT210	Bagdasarian *et al.*, 1981	Chloramphenicol	11.8	7.9×10^{-1}
pLAFR3	Staskwicz *et al.*, 1987	Tetracycline	22	4.2×10^{-7}

Table 3-5 Electroporation of *A. vinelandii* strain VK20 with various plasmids. Plasmid DNA (100 ng) was mixed with 1.52×10^{8} cfu/ml under the following electroporation conditions : 2.5 kV of voltage, 200 Ohms of current and 25 μF capacitance.

Categories	Strain UW (cfu/ml)[a]	Frequency of UW transformants per viable cell	Strain VK20 (cfu/ml)[a]	Frequency of VK20 transformants per viable cell
No DNA	6.8×10^{7}	N/A	5.6×10^{7}	N/A
Linear pQF50	2.37×10^{6}	3.5×10^{-2}	3.3×10^{7}	5.9×10^{-1}
Circular pQF50	4.6×10^{5}	6.8×10^{-3}	2.01×10^{7}	3.6×10^{-1}

a - cfu/ml determined after electroporation. Starting density before electroporation was about 1.5×10^{8} cells/ml.

Table 3-6 Comparison of number of transformants obtained on electroporating UW and VK20 with linear or circular plasmid DNA (pQF50).

was transformed with the plasmids pM112 and pMPCR2 and grown in high-iron and low-iron medium. Unfortunately, the growth of the transformants in low-iron medium was never sufficient to allow the determination of β-galactosidase activity as regulated by Fur in a homologous background.

3.10 Phenylalanine t-RNA Synthetase Assay

3.10.1 Aminoacyl t-RNA synthetases

The t-RNA synthetases belong to a class of essential enzymes which catalyze the first step of protein synthesis by covalently linking an amino acid to its cognate tRNA (Schimmel & Soll, 1979). The reaction is a two-step process and requires ATP :

$$\text{Amino acid} + \text{ATP} \longrightarrow \text{AMP-amino acid} + \text{PPi}$$

$$\text{AMP-amino acid} + \text{tRNA} \longrightarrow \text{tRNA-amino acid} + \text{AMP}$$

The high-energy bond of ATP is hydrolyzed in the first step to form an activated aminoacyl adenylate complex and PPi. In the next step, the amino acid is transferred to the 3' end of the tRNA, forming "charged" tRNA with the release of AMP (Meinnel *et al.*, 1995). A widely accepted assay method for measuring t-RNA synthetase activity is to measure the aminoacylation of t-RNA using radioactive amino acid substrates (Eigner & Loftfield, 1974).

3.10.2 Assay of Phenyalanine tRNA synthetase activity

In order to determine if the binding of Fur protein to the iron-box present within the *pheS* gene, regulates the *A. vinelandii pheST* operon, phenylalanine t-RNA synthetase (PheST) activity was measured in cells from low and high iron growth conditions. Radioactive phenylalanine (^{14}C-Phe) was used as the substrate and the enzyme activity present in the S-160 extracts of *A. vinelandii* cells was measured. A comparison of the rate of enzyme activity present in S-160 extracts of *A. vinelandii* UW strain grown in low and high iron condition, showed a difference of 1.4-1.6 fold (Fig. 3-32A and Fig. 3-32B). This result showed that the *A. vinelandii* phenylalanine t-RNA synthetase activity is up-regulated by iron availability although quite modestly.

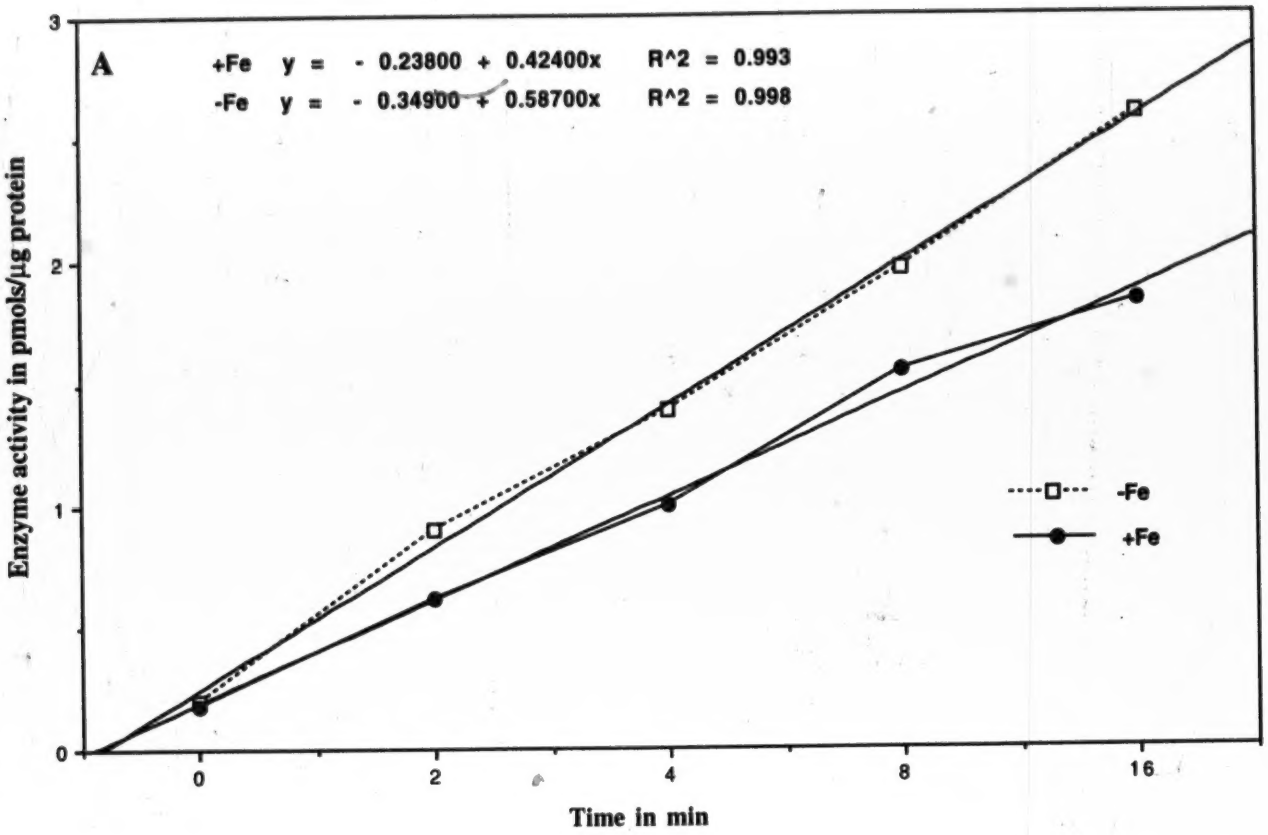


Fig. 3-32A Phenylalanine t-RNA synthetase activity in *A. vinelandii* UW grown under low and high iron conditions. The calculation of slope is shown in the inset.

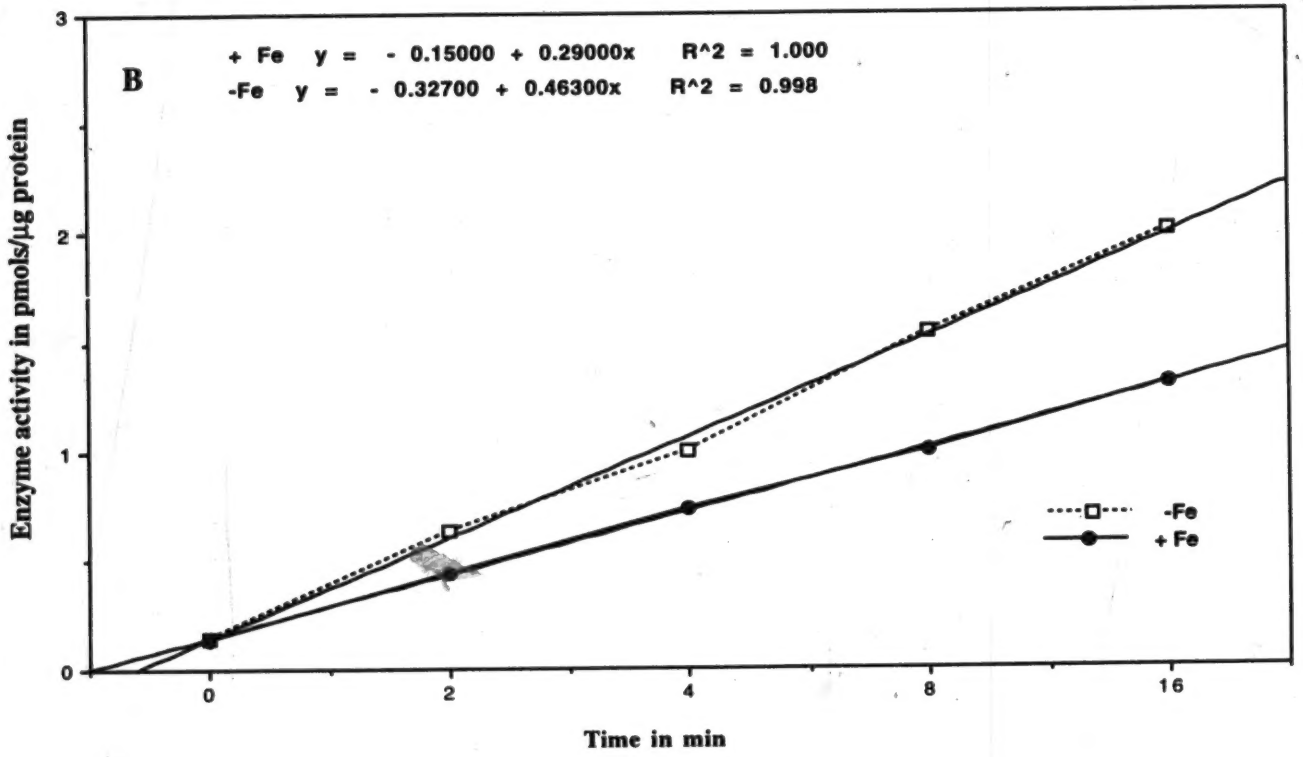


Fig. 3-32B Replicate assay of phenylalanine t-RNA synthetase activity in *A. vinelandii* UW, grown under low and high iron conditions. The calculation for the slope is shown in inset.

Chapter 4
Discussion and Conclusions

The phenotype of *A. vinelandii* strain UA22 was not known before this study was undertaken. Strain UA22 was characterized by its iron-regulated bioluminescence, which implied that the Tn*5*-*luxAB* cassette was inserted into the chromosomal DNA of UW downstream of an active iron-regulated promoter. The bioluminescence in strain UA22 was first visible after 15 h in low-iron medium, near the onset of azotobactin production. Since the strain was bioluminescent and this activity was iron-regulated, the first logical step was to look at the siderophore production by this strain. However, as shown in section 3.1, the production of azotobactin by strain UA22, was slightly impaired compared to UW. It was later found that the decrease in the production of azotobactin was affected by the presence of kanamycin not only in strain UA22, but also in other Tn*5* mutant strains of *A. vinelandii*. Thus, the phenotype of UA22 is apparently not directly linked with azotobactin production.

To gain an insight into the location of the Fe-repressible mutation, Dr. M. V. Woestyne constructed an *A. vinelandii* UA22 genomic library in *E. coli*. A clone containing ≈ 23 kb of UA22 DNA with the iron-regulated promoter and the Tn*5*-*luxAB* reporter genes was isolated. This clone (pMVW31) was further subcloned to obtain pMVW98, which contained the iron-regulated promoter on a 1.3 kb fragment.

The sequence of this DNA fragment plus 228 bp of upstream sequence was determined and, when compared with the sequences in the gene bank, showed a high degree of homology with the *pheST* operon region of several organisms, including *E. coli*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Mycobacterium leprae*. The sequenced portion also contained ≈232 bp of *luxA* sequence which was 100% identical with the *luxA* gene sequence of *Vibrio harveyi*. The *E. coli pheS* gene is a part of a cluster of genes comprised of: *thrS* coding for threonyl t-RNA synthetase, *infC* coding for initiation factor IF3, *rplT* coding for ribosomal proteins L20, followed by *pheS* and *pheT* coding for the α and β subunits of phenylalanine tRNA synthetase respectively, and *himA* which codes for the large subunit of the integration host factor required for the site-specific integration and recombination of bacteriophage λ (Grunberg-Manago *et al.*, 1984). The enzyme phenylalanine tRNA synthetase charges the tRNA with the amino acid phenylalanine and ribosomal protein L20 is the primary 23s rRNA binding protein. Both *rplT* and *pheS* are essential genes in an organism, as their gene products play a vital role in the protein synthesis machinery and are these genes are expected to be conserved in bacterial families. Hence, it was not surprising that the sequenced DNA of strain UA22 showed homology to the *pheS* gene from so many different microorganisms.

According to the homology search, the 1.541 kb DNA of strain UA22 contains 213 nucleotides (amino acids 47-117) of the *rplT* gene, but is missing the first 138 nucleotides (46 amino acids) of this gene. This is followed by an inverted repeat, which is believed to act as a Rho-independent transcription terminator in *E. coli* (Fayat *et al.*, 1983; Springer *et al.*, 1985). The *pheS* gene that is 0.99 kb long in *E. coli* is only 0.899 kb long in *A. vinelandii* UA22, as it is interrupted at its 3' end by the Tn*5-luxAB* insertion. In *E. coli*, the intergenic region between the *rplT* and *pheS* genes is 450 bp. However, in *A. vinelandii* UA22, this intergenic region was found to be only 192 bp long. In *E. coli*, the *pheST* genes are co-transcribed from a promoter situated 368 bp upstream of *pheS*. Features characteristic of an attenuation controlled mechanism are observed in the region between the promoter and the start of the *E. coli pheS*. These elements include an ORF of 14 amino acids with five phenylalanine codons and four sequences that are compatible with the formation of secondary structures on the transcript. The leader mRNA can fold to form a structure similar to a Rho-independent terminator (attenuator) or can form an alternative structure (antiterminator) precluding the formation of the attenuator (Fayat *et al.*, 1983).

In *A. vinelandii* UA22, there was no ORF, no phenylalanine rich region, and no sequence capable of forming secondary structures in the transcript apparent in the intergenic region between *rplT* and *pheS* genes. Similarly, no attenuator-like structures were identified in the *Thermus thermophilus pheS* operon (Keller *et al.*, 1992) where an imperfect inverted repeat 215-263 nucleotides upstream of *pheS* was found, but no open reading frame for a leader peptide overlapping the inverted repeat was detected. Thus, in *T. thermophilus*, there is no apparent attenuation control of the *pheST* operon and it may be expressed constitutively or be regulated in some other manner (Keller *et al.*, 1992). Another interesting case is that of *Bacillus subtilis*, where the transcription start site of the *pheS* gene is located 318 nucleotides upstream of the translational start (Brakhage *et al.*, 1990). There are two regions in this 318 nucleotide leader which exhibit dyad symmetry. The second inverted repeat is followed by a T-rich sequence resembling a Rho-independent transcription terminator, but the first region of dyad symmetry is upstream of this potential terminator and there is no indication of a phenylalanine-rich leader peptide that could modulate transcription.

In vitro transcription experiments with the *E. coli pheST* operon have shown that 90% of the transcription product initiated at the *pheST* promoter (p3 in Fig. 3-9) terminates at the Rho-independent terminator situated in front of *pheS* (t3 in Fig. 3-9). However, long runoff transcripts proceeding through the terminator and covering the *pheS* structural gene have also been observed. No other transcription initiation site has been detected between

the terminator and the *pheS* structural gene. It was discovered that 30% of the transcripts covering the *pheST* operon originate from the upstream gene *rplT*, the terminator of which is very inefficient (Springer *et al.*, 1985). The *pheST* genes are essential for bacterial growth and the absence of these genes cannot be compensated by supplementary nutrients in the growth medium. Therefore, even in the repressed state, phenylalanine tRNA synthetase must be present at a relatively high cellular concentration. This implies that the level of transcription traversing the terminator of the attenuator must be appreciable. It appears that the high level of termination at the attenuator is compensated for by the strength of the *pheST* promoter and by the presence of additional transcripts originating from upstream genes (Fayat *et al.*, 1983).

In the strain UA22 DNA sequence, there is an inverted repeat following the end of the *rplT* gene. However, no apparent promoter could be detected in the intergenic region between *rplT* and *pheS*. Nevertheless, cloning work had shown that a promoter was located in the 1.3 kb sequence between *rplT* and *luxA* DNA. Promoter probe studies revealed that the only detectable promoter activity was in the construct containing the internal *Hin*dIII-*Sal*I fragment of the *pheS* gene (pM112).

Promoter activity from the *Hin*dIII-*Sal*I fragment as measured by the β-galactosidase reporter gene was found to be up-regulated 2-2.5 fold in low iron conditions, suggesting the presence of an iron-regulated promoter in the cloned UA22 DNA. It was quite surprising to find promoter activity and iron-regulation within the *pheS* coding region of strain UA22. To explore the possibility of such a situation existing in *E. coli*, a fragment of the *E. coli pheS* gene lacking the upstream promoter and attenuator region was subcloned into the same promoter probe plasmid as was used for the *A. vinelandii* UA22 studies. This recombinant also possessed some promoter activity and β-galactosidase activity was found to be up-regulated about 2 fold in low iron conditions. The above facts lead us to believe that the *pheS* gene of *A. vinelandii*, as well as of *E. coli*, contains an iron box within its coding region and that there could be an active operator for Fur binding regulating the expression of downstream genes.

As mentioned earlier, *pheST* genes are essential for bacterial growth and the absence or mutation in these genes cannot be compensated for by supplementary nutrients in the growth medium. In *E. coli*, all tRNA synthetases (with the exception of lysyl tRNA synthetase) are encoded by a single copy of the gene (Martinis & Schimmel, 1995 & 1996; Saluta & Hirshfield, 1995). Thus, the existence of an insertional mutation in UA22, was surprising since in *E. coli* such a mutation would be lethal. The mutations which have been selected in enteric bacteria are those which affect the affinity of PheS for substrate

analogues or are temperature-sensitive mutants (Grunberg-Manago, 1987; Kast & Hennecke, 1991). The results of Southern analysis provided an explanation. It was found that strain UA22 contains mostly wild type copies of the chromosome and hence functional *pheST*, with very few mutant copies bearing the Tn*5-luxAB* disrupted *pheS* gene. Such an insertion mutation is possible only because of the polyploid nature of *A. vinelandii*. A similar observation has been made by Phadnis *et al.* (1988), where they noticed that only a fraction of the total chromosomes of *A. vinelandii* were tagged with Tn*10* or Tn*3* after mutagenesis. They found that cultivation of the cells in the presence of antibiotic increased the copy number of the tagged chromosomes, since these offered a selective advantage to daughter cells. However, when strain UA22 was grown in increasing concentrations of kanamycin, no apparent increase in the copy number of chromosomes carrying mutant *pheS* was observed. This result was observed presumably because the decrease in wild-type *pheS* copies did not present any advantage to the cells. The high level of genetic redundancy in *A. vinelandii* has always been a hindrance in obtaining homozygous mutants (Ramos & Robson, 1985; Contreras & Casedesus, 1987; Phadnis *et al.*, 1988).

The gel-retardation assays demonstrated that the *E. coli* Fur protein can bind to the *Hin*dIII-*Sal*I fragment of strain UA22 DNA. This implies that there is a functional iron-box within the fragment and that a heterologous Fur protein can recognize and interact with it. It is this attribute that allowed the original screening and cloning of iron-repressible Lux activity in *E. coli*. By scanning for the most conserved bases in the 19 bp consensus iron box sequence (ATT at positions 14, 15, & 16), one putative iron-box was located on the sense strand which showed approximately 53% identity with the consensus and another putative iron-box was located on the antisense strand, showing approximately 58% identity with the consensus and overlapping the iron-box on the sense strand. A 265 bp fragment of strain UA22 DNA containing these iron-boxes, was amplified by the polymerase chain reaction and the *E. coli* Fur protein was shown to bind this fragment and retard its mobility on a polyacrylamide gel. When cloned into the promoter probe vector pQF50 this PCR fragment drove the expression of the promoterless *lacZ* in the vector. The β-galactosidase activity in this construct (pMPCR2) was up-regulated 2-2.5 fold under low-iron conditions implying that the PCR fragment contained the functional iron-box and the promoter.

To obtain insight into the promoter activity residing in this PCR fragment, transcriptional analysis was done. The results of Northern blot analysis were inconsistent with an expected transcription start from within the *pheS* gene.

An attempt was made to map the transcription start site (if any) within the *pheS* gene by primer extension analysis. In *E. coli* JM106 (pMPCR2) RNA samples, a very faint cDNA

band was visible which corresponded to the start site at a C or T nucleotide (position 826 or 827). No cDNA was apparent in the RNA sample isolated from the negative control *E. coli* JM106 (pQF50). There was a lot of background noise in the primer extension analysis which did not disappear even after changing the assay conditions. No difference in the primer extended product could be seen in the samples grown under low-iron and high-iron conditions. Primer extension analysis of RNA isolated from strains UW and UA22 grown in low-iron conditions gave the same pattern and the primer extended product was found in the same position (a C or T at position 826 or 827).

Typically, an iron-box overlaps the -10 or the -35 promoter determinants (de Lorenzo *et al.*, 1987). The positioning of the iron-box helps the Fur protein to inhibit transcription initiation by binding to its target DNA (operator) and thereby inhibiting the activity of RNA polymerase at the cognate promoter. Examination of the sequences immediately upstream from the putative +1 site, revealed the presence of a sequence with some similarity to *E. coli* σ^{70} promoter determinants. The putative initiation site was positioned 9 nucleotides from the hexameric sequence GACGAT which shared only 3 out of the 6 most highly conserved nucleotides with the *E. coli* σ^{70} –10 promoter consensus sequence TATAAT (Hawley & McClure, 1983). Seventeen nucleotides upstream from the Pribnow-like sequence was the hexamer TCGCCG that also shared only 3 out of the 6 nucleotides with the *E. coli* σ^{70} –35 consensus sequence TTGACA (Hawley & McClure, 1983). The existing database of *Azotobacter* promoters is relatively small compared with that of *E. coli* and the best characterized *Azotobacter* promoters are from the nitrogen-regulated genes that are dependent on the σ^{54} rather than σ^{70}. Hence, it is difficult to be absolutely certain that the sequences predicted above are the real –10 and –35 promoter determinants for the transcript starting within the UA22 *pheS* gene. Furthermore, the results of primer extension analysis did not reveal any difference in the primer extended products obtained from samples grown in low-iron and high-iron conditions. Hence, there is no definite proof by this method, of a transcription starting at position 826/827 *in vivo*.

If the predicted internal *pheS* promoter is functional *in vivo*, it may help promote the transcription of downstream genes. In *E. coli, pheS* and *pheT* are believed to be cotranscribed from the same promoter present upstream of *pheS*. However, in strain UA22 no promoter activity was detected in the intergenic region between *rplT* and *pheS*. Therefore, this internal promoter (if functional) may be involved with transcription of the downstream *pheT* gene, which in *E. coli* does not have its own promoter. However, according to the results of Northern blot analysis, there is no definite evidence for the presence of a transcript arising from within the *pheS* gene of *A. vinelandii* UA22.

According to the *E. coli* attenuation control model, *pheS* and downstream *pheT* transcription is increased (by 2.3 fold) when phenylalanine availability is decreased (which results in a decrease in the concentration of phenylalanyl-tRNA). This ensures that $tRNA^{phe}$ will be formed and protein synthesis will continue despite diminished supplies of phenylalanine. However, *A. vinelandii* would rarely encounter an abundance of amino acids in its natural soil habitat. Although this organism can transport amino acids, its ability to transport phenylalanine is particularly weak (Mishra *et al.*, 1991). The internal supply of phenylalanine may always be limited by the biosynthetic capability of the cell, so that an attenuation control mechanism in the *pheST* operon of *A. vinelandii* would not offer an advantage. Also, since no attenuator-like sequences were apparent in *A. vinelandii*, this form of control is unlikely. However, during iron-limited growth the demand for these limited resources is expected to increase, as phenylalanine is directed into catecholate siderophore biosynthesis (Foster *et al.*, 1994). Thus in an iron-limited environment, as is frequently encountered by *A. vinelandii* in the soil, increased transcription through *pheS* can occur after the dissociation of a Fur-like protein from the internal *pheS* iron-box, to ensure that tRNA charging is competitive with other demands on the phenylalanine pool.

The results of phenylalanine t-RNA synthetase assay using radioactive phenylalanine and S-160 extracts of *A. vinelandii* UW cells grown in low-iron and high-iron conditions revealed that the enzyme activity was up-regulated 1.4-1.6 fold in low iron medium. Thus, the iron-box present within the *pheS* may help to fine-tune the regulation of *pheST* genes.

Promoter probe studies using the *E. coli pheS* gene also suggest that there is an internal iron-regulated promoter. The promoter probe studies were positive, but earlier workers did not find any transcript arising from within the *pheS* gene (Fayat *et al.*, 1983; Plumbridge & Springer, 1980; Springer *et al.*, 1983). This promoter may be most important and functional in iron-limited cells after the dissociation of Fur, to allow unimpeded transcription of *pheST*. Since it is within the *pheS* gene, it may not actually be the start of transcription, hence no "new" transcript initiates at this point. The real start of the *pheST* transcript in *E. coli* is upstream of *rplT*. Iron-limited *E. coli* cells will similarly divert phenylalanine into catecholate siderophore synthesis with the depletion of aromatic amino acid pools (Foster *et al.*, 1994) and increased aromatic amino acids biosynthesis (McCray, 1976). This additional control may be important when phenylalanine levels are relatively high and attenuation is in operation. Under these conditions, only 15% of the RNA polymerase molecules can read through the attenuator to the *pheST* genes (Grunberg-Manago, 1987; Plumbridge & Springer, 1980). The *pheS* internal promoter could provide a site for RNA polymerase binding to ensure transcription of downstream genes. On the

contrary, if the internal promoter is functional *in vivo*, it would help in transcription of the downstream *pheT* gene, but transcribing more of the *pheT* gene alone would not serve any purpose to the cell. Hence, this is speculation; whether the internal promoter identified in this study is functional *in vivo* is still a question to be answered.

The iron-box within the *A. vinelandii* UA22 and *E. coli pheS* genes is sufficiently homologous to the consensus sequence to allow the *E. coli* Fur protein to act as a repressor of β-galactosidase activity in pM112, pMPCR2 and pMKSS constructs and to retard target DNA migration in the gel binding assays. However, the regulation was not very strong and the reporter β-galactosidase activity was up-regulated only by 2.5 fold. The 19 bp consensus iron-box has two halves organized around a central A [5'- GATAATGAT A ATCATTATC-3']. On comparing the UA22 iron-boxes it was revealed that the UA22 iron-box had 3 out of 6 very highly conserved (in bold) residues all in the second half, whereas the *E. coli* iron box had 4 out of 6, one in the first half and 3 in the second half. This fits into the picture very well, as it is expected that expression of an essential gene like *pheST* would not be very tightly regulated by iron. Certain minimum levels of the *pheST* genes have to be maintained in the cell at all times, so a fine control mechanism mediated by the presence of iron and Fur may help the bacterium to decrease *pheST* expression when internal competition for phenylalanine is not so great.

Why would fine control of aromatic amino acyl tRNA be desirable? It is well known that under iron-limited conditions, there is a greater need for aromatic amino acids for the synthesis of siderophores and other aromatic compounds like ubiquinones. Hence, an increased ability to synthesize or to take up the aromatic amino acids may give the cell a growth advantage. The synthesis of siderophores would likely impose an imbalance on the aromatic amino acid biosynthetic pathway by depleting the chorismic acid pool. Together with this, there is also a competition for amino acids to maintain protein synthesis (Rosenberg & Young, 1974).

Iron restricted *E. coli* cultures have also been shown to have an enhanced capacity to transport aromatic amino acids into the cell (Buck & Griffiths, 1981). In fact, it has been shown that *E. coli* cells when grown in presence of ovotransferrin, an iron-binding protein, incorporates more phenylalanine into protein as compared to the cells growing under iron-replete conditions (Buck & Griffiths, 1981).

Iron is involved in biosynthesis of several aromatic amino acids including phenylalanine (McCandliss & Herrmann, 1978). It is an essential component of the enzyme DAHP synthase (7-phospho-2-keto-3-deoxy-D-arabino-heptonate D-erythrose-4-

phosphate lyase), where one mole of iron is present per mole of enzyme (McCandliss & Herrmann, 1978). The enzyme DAHP synthase catalyses the first committed step in the biosynthesis of aromatic compounds in bacteria and plants (Srinivasan & Sprinson, 1959). It has been shown that the total DAHP synthase activity is derepressed in extracts of cells grown in media chemically deficient in Fe^{+3} (McCray & Herrmann, 1976). Another enzyme, prephenate dehydratase, in the terminal pathway leading to biosynthesis of phenyalalanine, was also found to be derepressed at least 10 fold in extracts of cells grown in iron-deficient conditions (McCray & Herrmann, 1976). This enzyme is negatively regulated by its own product phenylalanine. If there is sufficient amount of phenylalanine in the cells, the enzyme prephenate dehydratase is repressed. Addition of phenylalanine to iron-starved cells brings the levels of DAHP synthase and prephenate dehydratase back to almost normal levels (McCray & Herrmann, 1976). Growth of *E. coli* in an Fe^{+3}-deficient medium causes accumulation of several abnormal species of amino acyl tRNAs including an undermodified version of phenylalanine-tRNA (Wettstein & Stent, 1968). It was shown that Fe^{+3} is involved in the enzymic methylthiolation of isopentenyl adenosine adjacent to the 3' end of the anticodon of tRNA (Wettstein & Stent, 1968; Rosenberg & Gefter, 1969).

In addition, growth of *E. coli* in an environment made low in free iron (by using chemically defined media or using media supplemented with unsaturated iron-binding proteins like transferrin, lactoferrin or ovotransferrin), results in accumulation of undermodified tRNA species which lack the methylthio group at the 3' end. The failure to fully modify the tRNA is believed to be a specific adaptive response to growth in an iron restricted environment (Griffiths & Humphreys, 1978; Griffiths *et al.*, 1978). It has been observed that *E. coli* grown in Fe^{+3}-deficient medium contains 90% - 95% of $tRNA^{phe}$, $tRNA^{trp}$ and $tRNA^{tyr}$ in the undermodified form (Griffiths & Humphreys, 1978). The loss of methylthiolation in the tRNA did not have a significant effect on aminoacylation. However, in *in vitro* polynucleotide synthesis directed by polyU, the altered $tRNA^{phe}$ was found to be only 20-30% as efficient as the normal $tRNA^{phe}$ (Buck & Griffiths, 1982). The altered $tRNA^{phe}$ translated polyUC with 40-50% efficiency of the $tRNA^{phe}$ from an iron-rich culture (Buck & Griffiths, 1982). This physiologically mediated change in post-transcriptional modification of tRNA leads to relaxation of transcription termination at the attenuator of certain operons of the aromatic amino acid biosynthethic pathway and thus to their increased expression under iron-restricted conditions (Buck & Griffiths, 1981). This depends upon the reduced translational efficiency of the undermodified tRNAs when reading contiguous codons (which are present in the leader peptide of the operator region of

the aromatic amino acid biosynthetic operon) (Buck & Griffiths, 1981; Eisenberg *et al.*, 1979).

The phenylalanine biosynthetic operon in *E. coli* is negatively regulated by its product, phenylalanine. This negative regulation is imposed through attenuation; when phenylalanine is less available, there is derepression of the biosynthetic operon. This derepression can also be imposed by iron-limitation by virtue of the decrease in the total phenylalanine pool in the cell (as phenylalanine is diverted towards synthesis of aromatic compounds and siderophores) or by undermodified $tRNA^{phe}$. The *pheST* operon in *E. coli* is also regulated by the attenuation mechanism and the operon is derepressed 2.3 fold during phenylalanine-depletion (Springer *et al.*, 1983).

Considering the above facts, it is not surprising that this study found that *pheST* expression is controlled by iron levels in the growth medium. Since the iron-box was found to be within the gene and the β-galactosidase fusion experiments showed that the activity was upregulated modestly by 2 fold under low-iron conditions, it can be surmised that the control is not very tight. This is to be expected since there are several reports in the literature on the presence of operators sites within a gene, all of which seem to be less tightly regulated as compared to the sites present near the promoter region (Collado-Vides *et al.*, 1991; Gralla & Collado-Vides, 1996). A good example is the *purR* gene, which codes for the repressor protein PurR. PurR is involved in regulating the *pur* regulon, consisting of genes responsible for the synthesis of purines (Rolfes & Zalkin, 1990a; Meng *et al.*, 1990). The *purR* gene is autoregulated and has two operator (O) sites both located downstream of the promoter. O1 is between +96 to +111 and O2 is between +184 and +199. The PurR repressor binds independently to the two operator sites. Operator site O2, which is located within the coding region of the *purR* gene, binds the repressor *in vitro* with a six-fold lower affinity than the operator site O1 (Rolfes & Zalkin, 1990b). It has been observed that PurR preferentially saturates the operator site O1 before O2. Fusion studies of *purR-lacZ* have shown that the expression of β-galactosiase in such constructs is regulated by PurR by only 2-3 fold. If only O2 is present, the repression is only 1.5-fold. On the other hand, the operator site for the PurR repressor for another gene of *pur* regulon *purF* is located around -28 in the region close to the -35 and -10 promoter determinants. When fused with *lacZ*, *purF* expression is repressed about 28 fold by PurR (Rolfes & Zalkin, 1988). Another gene, *purB*, of the same regulon has an operator site for the PurR repressor 224 nucleotides downstream of the trancription start point within the coding region of the gene (between 63 to 68 amino acids). The *purB-lacZ* fusion was found to be regulated by PurR by only 3-fold (He *et al.*, 1992).

It has been suggested (Rolfes & Zalkin, 1990; He *et al.*, 1992) that when the repressor is bound >200 bp downstream from the promoter, it acts as a road block and obstructs or stalls the progress of the transcribing polymerase. It also seems that the position of the operator with respect to the promoter determines the degree of regulation; the further the operator sites are from the promoter, the weaker the regulation will be (Collado-Vides *et al.*, 1991; Gralla & Collado-Vides, 1996). Considering the example of *purR*, it is very likely that the up-regulation of β-galactosidase activity seen in low-iron conditons, when *lacZ* is fused with an internal fragment of *pheS*, is in fact due to an operator for Fur-binding within the gene and not because of a promoter. The results of Northern blot analysis and primer extension analysis provide sufficient proof that, there is no functional promoter present within the *pheS* gene of *A. vinelandii*.

In *A. vinelandii*, not much is known about the phenylalanine biosynthetic operon nor about the presence of undermodified tRNAs in iron-limited growth conditions. Undermodified $tRNA^{Phe}$ has been shown to be present in other bacteria like *Salmonella typhimurium*, *Klebsiella pnemoniae* and *Pseudomonas aeruginosa* besides in *E. coli* (Buck & Griffiths, 1982; McLennan *et al.*, 1981). So we can only speculate at this point that undermodified tRNAs can be accumulated in *A. vinelandii* as well. As mentioned earlier, Buck & Griffiths (1981) showed that *E. coli* cells grown under iron-limited conditions have higher ability to transport phenylalanine among other amino acids, and also there is more incorporation of phenylalanine into the protein as compared to the cells grown under iron-replete conditions. This indicates that there is some role of iron in regulating the activity of phenylalanine tRNA synthetase in *E. coli*.

In summary, the phenylalanine tRNA synthetase activity in *A. vinelandii*, can be upregulated 1.4 to 1.6 fold in low-iron conditions presumably by the Fur protein dissociating from the internal operator. The internal Fur-box can act to block the transcription elongation as in the case of the PurR repressor (Fig. 4-1).

These hypotheses have been made on the basis that a Fur-like protein exists in *A. vinelandii*. The results of Western blot shows for the first time that this is in fact the case and that the protein shares serological homology with the Fur protein present in *E. coli* and *P. aeruginosa*. An attempt has been made to isolate the *fur* gene from *A. vinelandii* using the *E. coli fur* gene (in pMH15; de Lorenzo *et al.*, 1987) as the probe (data not shown). Preliminary experiments have shown that the two genes are not very homologous at the DNA level and that a more specific probe needs to be designed to find the *fur* gene in *A. vinelandii*.

An attempt was made to transfer pMPCR2 into a *recA*⁻ strain of *A. vinelandii* (VK20) by electroporation, to study the regulation of β-galactosidase activity in the presence of homologous Fur. However, because of the poor growth of strain VK20, especially in iron-limited medium, efforts to detect β-galactosidase activity were unsuccessful. The inability to carry out DNA repair may make strain VK20 prone to mistakes in DNA synthesis. DNA damage may be higher in iron-limited cells, where the cells are unable to protect themselves from the generation of oxygen-free radicals due to decreased activity of iron-containing enzymes like superoxide dismutase and catalase (Coffman *et al.*, 1990) Transforming VK20 with the plasmid may have created more problems for this strain. There have been several reports on plasmid transformants of *A. vinelandii* being deficient in producing normal levels of siderophores as compared to the untransformed strains (Glick *et al.*, 1988). This may be due to metabolic overload caused by the need for plasmid maintenance in the transformed cells.

It will be important to determine the location of the upstream promoter of the *pheS* gene. That would give us a clearer picture of the regulation of *pheST* operon. As well, the entire sequence of the *pheS* operon, especially any downstream gene(s), in *A. vinelandii* would permit us to compare the organization of the *A. vinelandii pheS* operon with that found in other bacteria.

Very little is known about the biosynthesis of siderophores in *A. vinelandii*. To understand how this bacterium regulates the production of its siderophores in such a unique manner will require a great deal of effort. The findings of this study have given some surprising and very interesting insights which add another dimension to the control of gene expression by iron.

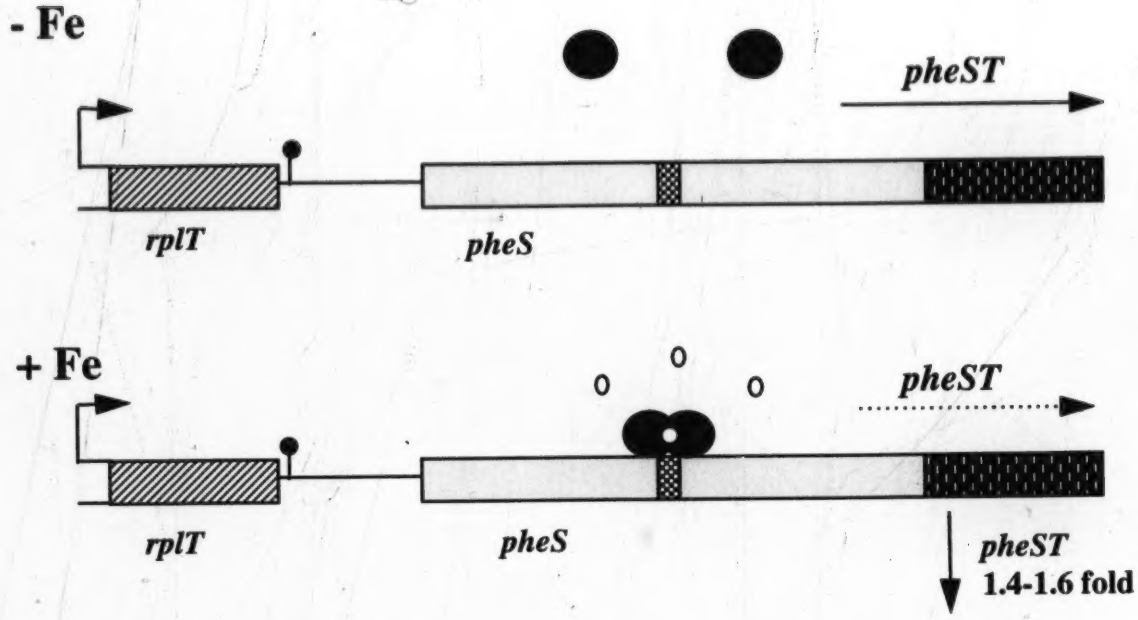


Fig. 4-1 Model showing the role of iron in the regulation of phenylalanyl-tRNA synthetase activity of *A. vinelandii*. Under iron-limited (-Fe) conditions there is normal expression of *pheST* genes, however in iron-sufficient (+Fe) conditions the Fur protein binds to the internal operator ( ) and represses the *pheST* expression by about 1.6 fold. Fur protein is designated by the big black balls and Fe^{+2} with small clear balls, bent arrow indicates the position of the possible promoter, horizontal arrow indicates the direction of the *pheST* gene expression and the lollipop indicates the position of the Rho-independent transcription terminator.

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